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THE ACTINOMYCETES

Vol. I

NATURE, OCCURRENCE, AND ACTIVITIES

by
Selman A. Waksman

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THE ACTINOMYCETES

VOL. I: NATURE, OCCURRENCE AND ACTIVITIES

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PREFACE

In any attempt to classify and divide living systems, nay, even living *versus* nonliving systems, certain borderline bodies are encountered which may be considered as transition forms from one group to another. This was recognized by the early students of the microscopic forms of life, who considered the bacteria and similar organisms as "protista" or primitive bodies, related, on the one hand, to the plants and, on the other hand, to animals. Recently accumulated information points also to viruses as transitory between nonliving and living bodies.

The actinomycetes form such a borderline system, but on a much more specialized scale. Considered by some as bacteria ("higher bacteria"), or Eubacteriales, and by others as fungi ("lower fungi"), or Hyphomycetes, actinomycetes are often placed in a group by themselves, with some of the properties of both. There are found, among the actinomycetes, certain forms that are more closely related to the bacteria and others that are nearer to the fungi.

My personal attention was first directed to the actinomycetes about 45 years ago. In 1914, as a senior in college, specializing in soil microbiology, or, as it was designated at that time, "soil bacteriology," I was assigned by my professor, Jacob G. Lipman, the task of making a comparative monthly study of the bacterial population of certain soil types located on the experimental grounds of the college. The results obtained in this study were used for a thesis which I presented the following June for my B.Sc. degree.*

Throughout the year 1914–1915, I sampled, at monthly intervals, several different soil types. Samples taken under sterile conditions were obtained from various depths (from the surface to 30 inches). These I brought to the laboratory and plated out, using suitable dilutions and proper culture media. After varying periods of incubation, I counted the colonies of bacteria developing on the plates. I was soon struck by the fact that a fairly large number of the colonies that I could observe did not look exactly like the majority of the others, more typical of bacteria. These particular colonies were compact and leathery in nature, pyramidal in structure, penetrating deep into the agar medium, frequently covered with a surface fuzz that was distinct from the substrate growth. On examination of such colonies even with a low-power microscope, the fuzzy growth proved to be made up of an aerial, branching mycelium that resembled that of fungus colonies.

When I brought the plates to my professor, he shook his head, smiled, and said, "Yes, I have been aware of the occurrence of these types of bacteria. Frequently they are designated as a special group, under the name actinomyces. You had better go and see our botanist, Professor M. T. Cook. He may be able to tell you more about them." Professor Cook was indeed familiar with the group, but merely as causative agents of potato scab. He considered them, not as bacteria but as fungi. He referred me to various papers in which further information could be obtained on this group of organisms. I decided in my very early studies, that the organisms could be differentiated from both bacteria and fungi. To my great satisfaction, I learned later that similar suggestions had already been made previously by others.

Thus, at the very threshold of my scientific career, I came in touch with a group of microorganisms that were to occupy a major part of my future scientific life. The final year of my undergraduate studies of these organisms was followed by three years of graduate work,† and by many more years as scientific assistant and finally as microbiologist at the New Jersey Agricultural Experiment Station.

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* "Bacteria, Actinomyces and Fungi in the Soil." Selman A. Waksman, Thesis, Rutgers College, New Brunswick, N.J., 1915 (Abstract published in J. Bacteriol. **1:** 101, 1916).

The following treatise is, in part, a summary of these investigations carried out for nearly half a century, mostly in the laboratories of Rutgers University, first at the College of Agriculture and Experiment Station, and more recently at the Institute of Microbiology. In a larger sense, however, I wish to give credit to the many other investigators who, by their careful and exhaustive studies, have so far advanced our knowledge of the actinomycetes during this first half o the Twentieth Century.

In the preparation of this volume, I have drawn freely from the various theses submitted by candidates for their Ph.D. degrees working under my direct or indirect supervision. I wish to acknowledge the assistance of my colleagues and collaborators, notably Dr. Ruth E. Gordon, Dr. Hubert A. Lechevalier, Mr. Robert A. Day, and Mrs Herminie B. Kitchen. I also wish to thank Dr. C. W. Emmons, of the National Institutes of Health, for reading Chapter 17, and Dr. L. A. Schaal, of the U. S. Department of Agriculture, for reading Chapter 18.

Selman A. Waksman

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† "Proteolytic Activities of the Soil Fungi and Actinomycetes," Selman A. Waksman, Ph.D. Thesis, University of California, December 1917 (J. Bacteriol. 3: 475–492, 509–530, 1918).

INTRODUCTORY

No other group of microbes, and for that matter no other group of living systems, whether of plant, animal, or microbial origin, has been in recent years the focus of so much attention by the investigator, especially the microbiologist, the chemist, and the medical scientist, and by the pharmaceutical manufacturer, as the actinomycetes. Only 20 years ago scarcely a dozen laboratories in the whole world were devoting much attention to this group of organisms, and they were concerned largely with either disease-producing or soil-inhabiting forms. Today, literally thousands of investigators in numerous laboratories throughout the world are isolating cultures of actinomycetes from soils and other substrates and studying their physiological and biochemical activities. This increased attention is due primarily to the discovery that the actinomycetes comprise many forms that have the capacity to produce a large number of chemical substances capable of inhibiting the growth of microorganisms, especially disease-producing forms. These substances have come to be known as antibiotics. The discovery that certain actinomycetes can produce growth-promoting substances or vitamins and certain potent enzyme systems has added greatly to this interest. Many of the antibiotics produced by the actinomycetes have found extensive practical application in the control of infectious diseases of man, animals, and plants; also in animal nutrition; and in the preservation of biological products, including virus preparations, and of human foodstuffs.

Our first knowledge of the actinomycetes dates back to 1875, when Ferdinand Cohn named an organism he found in the tear duct of the human eye *Streptothrix Foersteri*. This was soon followed (1877 to 1878) by a description by Harz, of another organism, *Actinomyces bovis*, found in "lumpy jaw" of cattle. Since then, many actinomycetes have been isolated, and a number of genera and hundreds of species have been described. These include organisms causing animal and plant diseases and numerous saprophytes occurring in soils, in dust, in water basins, and in other natural substrates.

Because of the above two generic names and for other reasons, the systematic position of actinomycetes became highly confused. Animal and plant pathologists, botanists, zoologists, mycologists, bacteriologists, and biochemists were eager to introduce new names in describing as new species freshly isolated cultures of actinomycetes. New genera and new species were thus created, without due regard to previously established names or even previous descriptions. This tended to complicate greatly our knowledge of the taxonomy and classification of the actinomycetes.

A number of subsequent milestones in the history of actinomycetes should be noted. Among them were the isolation by Israel of a pure culture of an anaerobic organism, for which the generic name *Actinomyces* was reserved; the introduction of synthetic media by Krainsky and by Waksman and Curtis; the recognition of the sporulating mechanisms of actinomycetes by Ørskov; the classification systems of Waksman and Henrici and of Krassilnikov; the isolation of antibiotics from cultures of actinomycetes; and finally the study of the cell walls of actinomycetes. These and numerous other milestones have marked the development of our knowledge of the actinomycetes from the original concept that they were a small group of negligible organisms causing certain obscure diseases to the comprehensive recognition that they represent a large and highly important microbial group of universal distribution, possessing numerous biochemical activities, and of great practical potentialities.

From an ecological point of view, the interest in the actinomycetes has centered largely upon the study of their occurrence in soils, in composts, in water basins, in the atmosphere, and in the infected tissues of living systems. Their role as causative agents of human, animal, and plant diseases at first attracted wide attention, but more recently this interest became of limited significance. Under some conditions, however, the actinomycetes may play a highly important role in the causation of certain plant diseases, such as potato scab.

From a biochemical point of view, interest in the actinomycetes has centered largely upon their role in the transformation of organic matter in the soil and their ability to form antibiotics, vitamins, and enzymes. The interest in the antibiotics produced by actinomycetes has been phenomenal. It all began with the isolation of actinomycin in 1940. This was followed by the isolation of streptothricin in 1942 and of streptomycin in 1943, and later of chloramphenicol, the tetracyclines, the erythromycins, the neomycins, novobiocin, oleandomycin, nystatin, and numerous others. To date, more than 500 different antibiotics have been isolated from cultures of actinomycetes. Many of them have been obtained in the form of pure compounds, the chemical nature of which has been determined. Others are still of unknown composition. Nearly 25 of these antibiotics have already found extensive practical application as chemotherapeutic agents. Of the total 2,400,000 pounds of antibiotics produced in the United States in 1955, valued at more than a half a billion dollars, at least two-thirds have been obtained from cultures of actinomycetes.

The interest in the antibiotics evoked tremendous interest in these organisms, their distribution in nature, their growth and nutrition under controlled conditions, and finally their biochemical activities. Among the earlier treatises devoted to the subject of actinomycetes, note should be taken of the work of Lieske (1919), Duché (1935), Kriss (1937), Krassilnikov (1938), and Cope (1938). I have personally contributed to many phases of the study of actinomycetes. Following my work on "The cultural properties of actinomycetes," published in 1919, I edited the section on actinomycetes in the various editions of *Bergey's Manual*, beginning with the first in 1923 and including the seventh in 1958. My more recent books include a book on *The Actinomycetes* published in 1950 and various volumes and papers on the antibiotics of actinomycetes.

The rapid accumulation of basic knowledge concerning the actinomycetes justifies a comprehensive treatise at this time. In this work, I have made no attempt to review or even to list the extensive literature on this subject. Only certain pertinent references have been selected. In view of the fact that more than 6000 references on the subject of a single antibiotic, streptomycin, had been collected (as of 1952!) one can readily imagine the extensive literature covering the other antibiotics that have found practical application in the treatment of numerous human and animal diseases, in animal feeding, and in the preservation of various biological preparations and food materials. And all of these antibiotic references, of course, would be in addition to the thousands of papers that have been published relating to the organisms themselves.

This treatise is limited to a review of our knowledge of the true actinomycetes. It does not concern itself with the various bacterial forms frequently included among the *Actinomycetales*, namely, the mycobacteria, corynebacteria, and mycococci.

In view of the frequent references to members of the various genera of the actinomycetes by vernacular designations, the following comments may be made here:

The terms "actinomycete" and "actinomycetes" will be used in this treatise as inclusive terms for any or all of the organisms now included in the Actinomycetales, exclusive of the mycobacteria and corynebacteria. The term "*Actinomyces*" will be used only when referring to the single genus of that name; "actinomyces" will be used as the vernacular expression only for members of this genus, both in singular and in plural senses. The term "streptomyces" will be used as a vernacular expression of the genus *Streptomyces*, both in singular and plural senses. The terms "nocardia" and "nocardias" will be used in the vernacular for members of the genus *Nocardia*, and "micromonospora" and "micromonosporas" for "*Micromonospora*."

TABLE OF CONTENTS

The Actinomycetes

VOLUME I

OCCURRENCE, NATURE, AND ACTIVITIES

Preface v
Introductory vii
1. Historical Background 1
2. Isolation, Identification, Cultivation, and Preservation 17
3. Distribution in Nature 29
4. Nomenclature and General Systems of Classification 47
5. Morphology, Cytology and Life Cycles 71
6. Variations, Mutations and Adaptations 95
7. Physiology—Growth and Nutrition 113
8. Mineral Metabolism 138
9. Biochemical Activities 148
10. Lytic Mechanisms 168
11. Production of Enzymes 183
12. Production of Vitamins and Other Growth-Promoting Substances 193
13. Production of Pigments 198
14. Antagonistic Properties 207
15. Production of Antibiotics 225
16. Decomposition of Plant and Animal Residues 243
17. Causation of Animal Diseases 251
18. Causation of Plant Diseases 265
Epilogue 277
References 278
Index of Organisms 296
Index to Species of Actinomycetes 319
General Index 322

CHAPTER 1

Historical Background

What Are Actinomycetes?

Actinomycetes are a group of branching unicellular organisms, which reproduce either by fission or by means of special spores or conidia. They are closely related to the true bacteria; frequently, they are considered as higher, filamentous bacteria. They usually form a mycelium which may be of a single kind, designated as substrate (vegetative), or of two kinds, substrate (vegetative) and aerial (in part sporogenous).

In the early descriptions, actinomycetes were often defined as "unicellular microorganisms, 1 μ in diameter, filamentous; branching monopodial, seldom dichotomous, producing colonies of radiating structure." Two forms of reproduction have commonly been recognized: (a) fragmentation, or oidia-formation, and (b) segmentation. Both kinds of spores grow in ordinary media to form a filamentous mycelium.

Frequently the actinomycetes have been looked upon as a separate group of organisms occupying a position between the filamentous fungi and the true bacteria. It has even been said that actinomycetes are the original prototypes from which both fungi and bacteria have been derived. Some forms of actinomycetes, such as members of the genus *Nocardia*, are known to have their counterparts among the bacteria; other forms, like some species of *Streptomyces*, *Micromonospora*, and some of the other genera, have their counterparts among the fungi. The similarity in diameter between bacteria and the mycelium and spores of actinomycetes and certain common chemical and biochemical properties, recently discovered, suggest that the actinomycetes should be classified with the bacteria. They are usually placed in a separate order, the Actinomycetales, which is said to be distinct from the Eubacteriales, or the true bacteria, although this relationship has recently been questioned.

The actinomycetes are generally recognized to represent a large and heterogeneous group of microorganisms, comprising several genera and numerous species. They vary greatly in their morphology, physiology, biochemical activities, and role in natural processes. They play an important part in the cycle of life in nature by bringing about the decomposition of complex plant and animal residues and the liberation of a continuous stream of available elements, notably carbon and nitrogen, essential for fresh plant growth. Some of the biochemical activities of the actinomycetes are now being utilized for the large-scale production of chemical substances essential for public health and human economy.

Early Concepts

The early history of the actinomycetes revolves around their role as causative agents of disease, especially a disease in cattle known as "actinomycosis" or "lumpy jaw."

Ferdinand Cohn's first description of an actinomycete was based upon his study of an

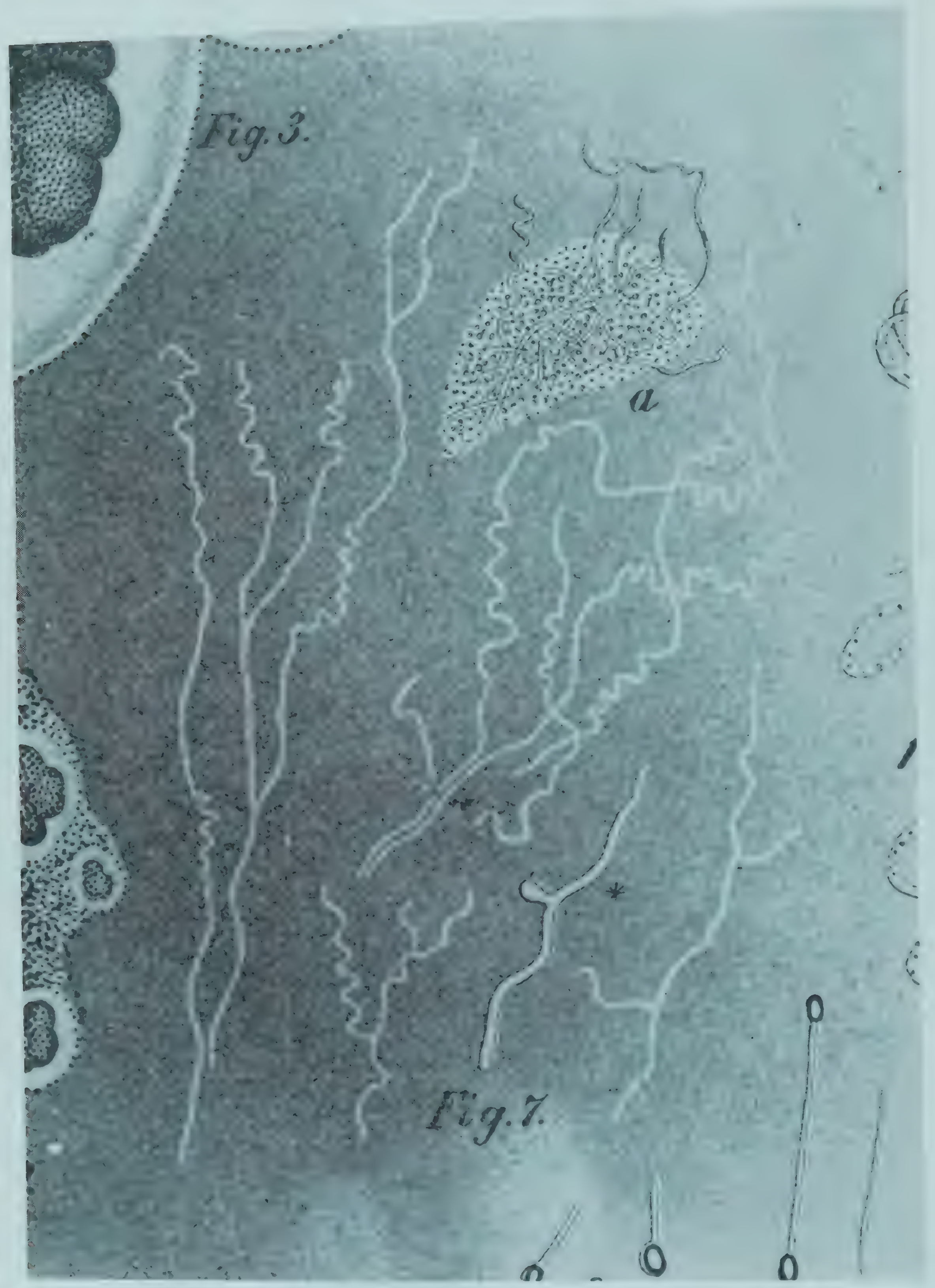

FIGURE 1. The first illustration of an actinomycete ever published, *Streptothrix Foersteri* (Reproduced from: Cohn, F. Untersuchungen über Bacterien II. Beitr. Biol. Pflanzen 1: 141–207, 1875).

organism found in concretions of the lachrymal ducts and which he named *Streptothrix Foersteri*. The concretions were transmitted to Cohn by Foerster for microscopic examination. Cohn says: "On April 15, 1874, he transmitted to me a mass which was whitish, like tallow, easily broken down and still consisting of fine, very thin colorless branching threads running parallel to one another or in various directions, curving and in places also wavy." This type of growth reminded Cohn of the curvatures of spirilla and spirochaetes, although it was more irregular. The threads were found to break up into fragments, some of which reached a length of 50 μ. The branching filaments were surrounded with masses of micrococci, filling the spaces between the threads. These filaments were distinctly different from the straight, thick, and unbranched (false-branching) *Leptothrix buccalis* commonly found in the mouth. The photographs of the organism published by Cohn leave no doubt that this was a true actinomycete. Cohn considered this organism to be a bacterial form with branching mycelium, though all attempts to cultivate the organism failed.

Two years later, Harz examined a pathologic specimen, obtained from "lumpy jaw" of cattle and submitted to him by Bollinger. He gave to the organism observed in this specimen the generic name *Actinomyces* and the specific name *bovis*. No pure culture was obtained. The masses of filaments were found to be arranged radially, which suggested the name "actino-myces" or "ray-fungus."

Neither of these two generic descriptions was universally accepted, largely because the first (*Streptothrix*) had been preempted in 1839 by Corda for a true fungus and the second (*Actinomyces*) had been meeting with much criticism, because the description of the organism was based on its etiology rather than on its morphology and cultural characteristics.

The first isolations of pure cultures of actinomycetes from human and animal infections involved some difficult problems in ecology and taxonomy. They were the primary causes of much confusion in the history of actinomycetes. O. Israel claims to have isolated in 1884, from a human infection, an aerobic filamentous organism, the hyphae undergoing ready fragmentation. Bostroem claims to have isolated in 1885, also from human cases, an aerobic, filamentous, spore-forming culture. Nocard isolated an aerobic culture in 1888 from an animal infection. This was followed (1889) by the isolation from a human infection of an aerobic culture by Afanassiev. In 1890, Eppinger isolated a nonsporulating aerobic organism, and Wolff and J. Israel isolated, the same year, a nonsporulating microaerophilic form.

These cultures came from different sources and, because of their filamentous nature, were considered to represent the isolates of Cohn and Harz. None of the above isolations were, however, the cause of as much confusion as the report made by Bostroem of his isolation in 1890 of a pure aerobic culture of an actinomycete from a case of actinomycosis. This culture, now known to be a *Streptomyces*, rapidly found a place among the various collections and was believed at first to be the true cause of actinomycosis. The general consensus now is that this culture did not represent the causative agent of the disease but was merely an air contaminant. Unfortunately, this error remained to plague the subsequent literature of the actinomycetes and became a cause of much confusion. First, the claim that *Actinomyces bovis* was an aerobe rather than an anaerobe was wrong; second, the wide distribution of the contaminant led many to assume that actinomycosis was caused by an aerobic organism similar to the group now designated as *Streptomyces*.

For many years, investigators continued to believe either that the causative agent of actinomycosis was an aerobe or that there were two forms, one an aerobe and the other an anaerobe. There is no doubt now that Bostroem never succeeded in growing the true etiologic agent of actinomycosis but that some of his attempted isolates became contaminated with saprophytic actinomycetes from the dust in the air, and thus resulted in the mistaken isolation. Topley and Wilson

(1929) proposed that this isolate be named *Actinomyces graminis*. Vuillemin (1931) considered it to be identical with *Actinomyces sulphureus* Gasperini (1894).

In the absence of pure cultures of the causative agent of the disease for comparative studies, some of the early workers on actinomycetes had only a limited concept of the growth and life cycle of these organisms. This is illustrated, for example, in the description by MacFayden (1889) of the history of an actinomycete colony:

"It has its starting point in one or more cocci transported by the plasma currents or by the agency of a carrier cell (leucocyte). The cocci multiply by elongation and subsequent fission. By elongation some of the cocci give rise directly to short bacillary forms, and through these to long filaments. The further extension of the colony is effected by the growth and multiplication of both threads and cocci. The majority of the threads tend to develop clubs at their outer ends (involution forms)." For more phantasy and inaccuracy, one would have to search widely in microbiological literature.

Not much progress in the general understanding of these organisms seems to have been made during the next 20 years, as illustrated by reference to them in the Second Edition of H. W. Conn's (1909) *Agricultural Bacteriology*. In speaking of the actinomycetes, he says:

"Under this head are included a few forms of fungi which resemble other bacteria in some respects, but differ in others. They are composed of threads which are commonly larger than the threads of bacteria, and which may show frequent branching, a characteristic not usual in bacteria. They also have a peculiar method of forming reproducing bodies. The group is not one of very great importance. One type of *Streptothrix* is extremely abundant in soil and appears as round, white opaque colonies with an extensive brown halo upon the plates."

An important cause of confusion was the fact that the actinomycetes were grown on nitrogen rich organic media, now known to be totally unsuitable for them to form a characteristic growth, essential for comparative studies and for proper identification. As a result, a highly complex terminology was developed for the designation of actinomycetes; numerous descriptions of "new" species soon began to appear. This is illustrated by the summary made, as early as 1892 to 1894, by Gasperini (Table 1). There is no wonder, therefore, that the nature and classification of the actinomycetes soon appeared hopeless.

The adoption of the name "actinomycetes" was suggested by Gasperini and Lachner-Sandoval. Sanfelice, impressed by the analogy of the biological properties of the actinomycetes and those of the tuberculosis organism, suggested that the relationship of the actinomycetes to the bacteria was closer than to the fungi. Gasperini emphasized that the species or varieties belonging to the actinomycetes, included under one genus *Actinomyces*, show great variations in form and in behavior, especially in their ability to produce aerial spores and soluble pigments. Some of these properties were recognized to be inconstant and were found to depend on the conditions of culture and the composition of the medium; minor variations of the latter could bring about marked changes in growth and pigmentation.

Historical Periods

Before we consider in detail the historical background of our knowledge of the actinomycetes, we must recognize certain distinct periods in which the various concepts concerning the nature of these organisms and their importance in the cycle of life became crystallized. There is, of course, considerable overlapping of the various periods, since no one period came to an end before another

TABLE 1

Species of actinomycetes recognized in 1892 to 1894 by Gasperini

Name	Observer	Name	Observer
Act. bovis sulphureus	Rivolta	*Act. bovis* (?)	—
Act. Foersteri	Cohn	*Streptothrix Foersteri*	—
Act. canis	Vachetta	*Act. pleuriticus canis familiaris*	Rivolta
		Act. canis	Rabe
Act. bovis farcinicus	Nocard	*Bacillus farcinicus*	—
Act. cati	Rivolta	—	—
Act. bovis albus	Gasperini	*Streptothrix* 1, 2, 3	Almquist
		Streptothrix Albus	Rossi-Doria
Act. asteroides	Eppinger	*Cladothrix asteroides*	—
		Strept. asteroides	Gasperini
		Strept. Eppingerii	Rossi-Doria
Act. chromogenus	Gasperini	*Strept. chromogenus*	—
		Strept. niger	Rossi-Doria
		Oospora Metschnikowi (?)	Sauvageau & Radais
		Oospora Guignardi (?)	Sauvageau & Radais
Act. bovis luteo-roseus	Gasperini		—
Act cuniculi	Schmorl	*Streptothrix cuniculi*	—
Act. Hoffmanni	Gruber	*Micromyces Hoffmanni*	—
Act. albido-flavus	Rossi-Doria	*Streptothrix albido-flava*	—
Act. violaceus	Rossi-Doria	*Streptothrix violacea*	—
Act. carneus	Rossi-Doria	*Streptothrix carnea*	—
Act. citreus	Gasperini	—	—
Act. pluricolor (?)	Terni	—	—
Act. arborescens	Edington	—	—
Act. ferrugineus	Naunyn	—	—

one began. These periods can be briefly outlined as follows:

1. *Causation of disease.* This period began in 1875 and continued to the end of the 19th century. The predominant interest in the actinomycetes during these years was in their role as pathogens, first in human and animal diseases, especially actinomycosis in cattle, and later in plant diseases, particularly potato scab (R. Thaxter).

2. *Occurrence and importance in soil.* During the next two decades, beginning about 1900, with the work of Beijerinck, and ending about 1919, with the work of Krainsky, Conn, and Waksman and Curtis, the interest in the actinomycetes was predominantly concerned with their occurrence in soils and in other natural environments. The introduction of synthetic media served to broaden greatly our knowledge of the nature and occurrence of the actinomycetes.

3. *Biological period.* Between 1919 and 1940, intensive knowledge accumulated concerning the cultural properties of the actinomycetes, their physiology, and their biochemical activities, notably their antagonistic effects upon bacteria and fungi. This period may be said to have begun with the work of Waksman in 1919 and Lieske in 1921. It continued with the studies of Gratia and his group on the bacteriolytic effects of certain actinomycetes and of Krassilnikov and his associates on the antibacterial properties of actinomycetes. Problems of variability (Schaal, Tempel, Kriss), decomposition of plant and animal residues (Conn, Waksman

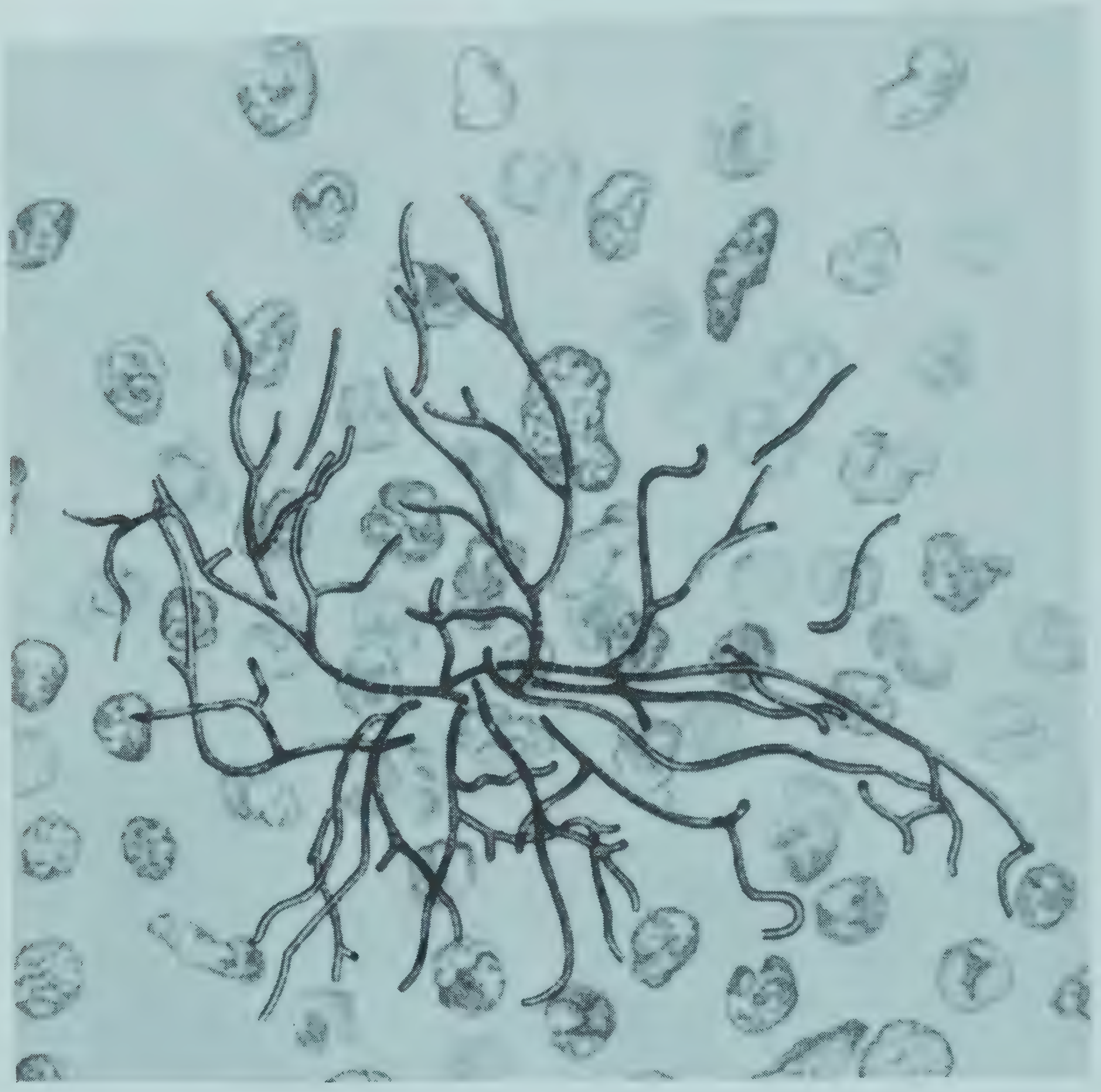

FIGURE 2. Growth of an actinomycete in animal tissue (Reproduced from: Butterfield, E. E. J. Infectious Diseases **2:** 430, 1905).

et al.), and the importance of actinomycetes in natural processes were given ever-growing consideration.

4. *The biochemical or, more precisely, the antibiotic period.* A new era in the study of actinomycetes began about 1940. Then, it was established that a large number of these organisms are capable of producing a great variety of chemical substances that have the capacity to inhibit the growth of various microorganisms, and that some of these substances can find chemotherapeutic applications in the treatment of numerous infectious diseases of man, animals, and plants. This resulted in extensive investigations devoted to the nutrition of actinomycetes, the biosynthesis by them of various chemical compounds, their chemical structure, life cycle, and numerous biochemical activities.

1. *Actinomycetes as Causative Agents of Disease (1875–1900)*

The initial work on the actinomycetes was done by two eminent botanists, F. Cohn in 1874 and C. O. Harz in 1877. Unfortunately, two circumstances soon shifted the interest in these organisms from the botanists to clinicians and veterinarians.

1. Cohn's work on actinomycetes, and for that matter on bacteria in general, was completely neglected by nearly all botanists following him. Even so outstanding a botanist as Roland Thaxter, who about 15 years later studied another group of actinomycetes, namely, the organisms causing potato scab, called them fungi (*Oospora scabies*), completely overlooking their close relationship to the bacteria.

2. The second circumstance had to do with the fact that the role of microbes as causative agents of infectious diseases had just come to be recognized as a result of the brilliant work of Louis Pasteur, Robert Koch, and numerous others. It was but natural that diseases caused by actinomycetes should also soon begin to attract attention. In 1876, Bollinger observed branching mycelium in the diseased jaw of a cow and recognized that a microbe was the causative agent of the disease. He handed this material to Harz, who examined the granules and observed the characteristic radiation, with the result described above. Simultaneously, J. Israel examined granules containing similar mycelium in two pathologic specimens of man; unfortunately, he was confused by the presence of secondary infections due to staphylococci. It was Ponfick, in 1879, who definitely established the role of actinomycetes as causative agents of human diseases. Israel's first clinical account appeared in 1885. Wolff working in collaboration with Israel soon established the anaerobic nature of the organism.

These pioneering studies were followed by the careful work of Gasperini and others who interpreted clearly the nature of the disease of actinomycosis and the role of actinomycetes in its causation.

The study of diseases caused by aerobic actinomycetes in animals and in man also began to receive attention, with the observations of Nocard and Trevisan. Unfortunately, the nature of the causative agents of these diseases and the complex nomenclature that soon evolved continued to cause confusion for years. As late as 1925, Dresel suggested that the term "actinomycosis" be reserved for those diseases that are caused by the anaerobe (*Actinomyces israeli*) and that another name be selected for the diseases caused by aerobes, in case the name "Streptothrix" should finally be disqualified.

Foulerton wrote in 1899 that the disease known as "actinomycosis" in cattle and man had long been recognized clinically to be caused by more than one species of actinomyces, infections themselves being very similar. Gasperini described three such varieties or species. Wolff and Israel isolated from human actinomycosis an organism, "a streptothrix fungus," which differed from "Streptothrix actinomycotica" in that the growth under anaerobic conditions was very free, whilst in the presence of oxygen it was very scanty. Levy isolated from five actinomyces cases in man an organism which resembled that of Wolff and Israel in its free growth under anaerobic conditions. Kruse recognized two species as causing actinomycosis: (1) "*Streptothrix actinomyces*" of Rossi-Doria, said to be an "aerobic fungus;" and (2) "*Streptothrix israeli*," an "anaerobic fungus." A number of other investigators de-

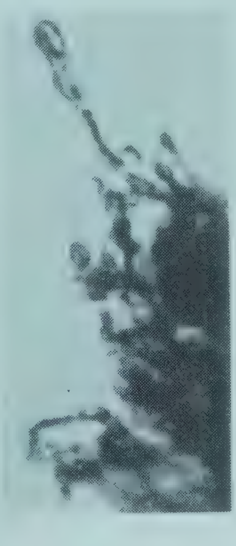

FIGURE 3. Club formation by a culture of *A. bovis* grown in human blood serum (Wright, J. H. J. Med. Research 13: 349–404, 1905).

scribed, according to Foulerton, cases "which clinically present the features of actinomycosis, but which are caused by parasites which differ sufficiently from streptothrix actinomycotica to entitle them to be regarded as separate species." Bruns noted a culture which he believed to be similar to that described by Berestnew as occurring in a case of "pseudoactinomycosis." Bruns objected to the use of this term and considered the organism in question to belong to a new species.

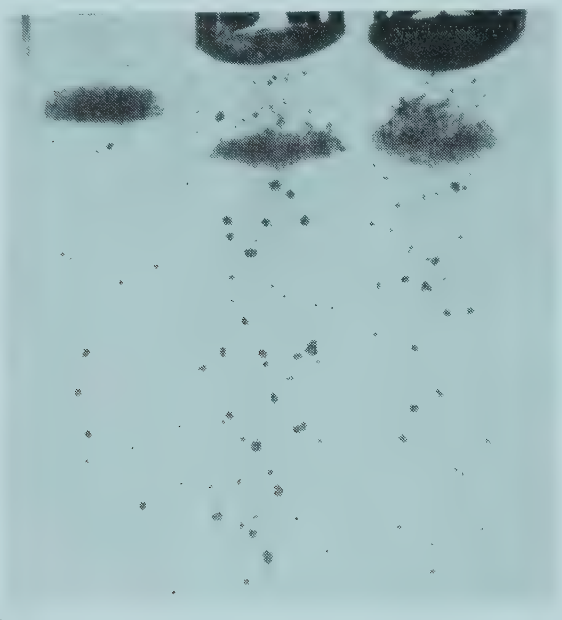

FIGURE 4. Appearance of cultures of *A. bovis* in agar tubes (Reproduced from: Wright, J. H. J. Med. Research 13: 349–404, 1905).

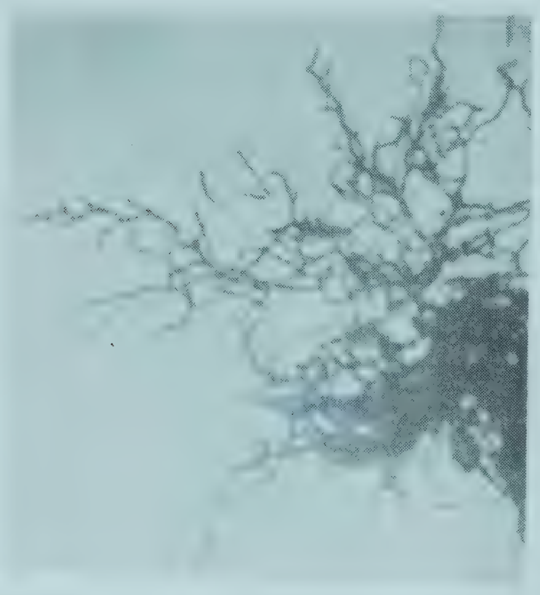

FIGURE 5. A cross-section of a colony of *A. bovis* in agar (Wright, J. H. J. Med. Research 13: 349–404, 1905).

Thus, the differentiation between aerobic and anaerobic forms as causative agents of specific diseases gradually became established, particularly through the work of Foulerton and Price-Jones (1902), Wright (1905), and others.

The first historical period is thus characterized by serious difficulties that were a direct result of the complications involved in the isolation and identification of the causative agents of disease conditions in animals and man, and by the problems of proper nomenclature, which will be discussed in detail in Chapter 4. Attention has already been drawn to the confusion introduced by Bostroem, in 1890, who isolated from infected lesions aerobic air contaminants, which he designated as the causative agent of the disease. Another cause of confusion was the introduction of the term "streptothricosis," based on Cohn's original designation, as a synonym—not always recognized as such—for "actinomycosis," or a disease caused by actinomycetes. Later suggestions that such names as "nocardiosis" and "maduramycosis" be used did not help to straighten out the ensuing confusion.

The study of the causation of plant diseases by actinomycetes also falls within this period. As has been noted, Thaxter elucidated, in 1891, the nature of the pathogenic organism concerned in potato scab. He called it *Oospora scabies*. The culture was isolated and carefully studied. This soon led to extensive investigations by numerous botanists and plant pathologists, which continued into the subsequent periods.

Outstanding work on the occurrence of actinomycetes, their morphology and systematic position, was also carried out during this period. It is sufficient to mention such names as Rossi-Doria, Lachner-Sandoval, and soon after Neukirch, and various other bacteriologists.

2. *The Soil Period* (*1900–1919*)

Just as the first period was initiated and greatly influenced by the work of Cohn and Harz, the second period may be said to have been initiated by the work of W. H. Beijerinck, on the role of actinomycetes in soil processes. It was soon followed by that of Hiltner and Störmer, on the actinomycete population of the soil. While studies on the pathogenicity and classification of actinomycetes continued during this period, as in the work of Sanfelice, Wright, and many others, ever-growing attention was being paid to the saprophytic actinomycetes, their physiology, and their role in nature, thus resulting in the broadening of our understanding of actinomycetes as a large and important group of microorganisms.

In 1900, Beijerinck published a paper on the activities of an organism belonging to the group of saprophytic actinomycetes, designated as *Streptothrix chromogena*. This organism belonged to the very large group of actinomycetes now included in the genus *Streptomyces*. Beijerinck attempted to throw light upon its physiology and its role in soil transformations. He pointed out that actinomycetes in general are omnivorous organisms, capable of living in an environment rich in organic matter as well as in a very poor environment. Even distilled water and an ordinary laboratory atmosphere are sufficient for the growth of some of these forms. Beijerinck emphasized, however, that actinomycetes are unable to carry out such processes as the fixation of atmospheric nitrogen, an ability ascribed to them later, on insufficient grounds, by others. He also found that actinomycetes produce, in glucose media, traces of acid, probably lactic, and that they are able to reduce nitrate to nitrite. It was suggested that, under certain conditions, the last process may lead to losses of nitrogen through the interaction of nitrites with ammonium compounds.

Beijerinck believed that the black pigment produced by certain actinomycetes on protein media might function as an oxidizing agent. This led him to postulate the significance of these organisms in natural processes. Actinomycetes were found to occur abundantly in the soil to considerable depths,

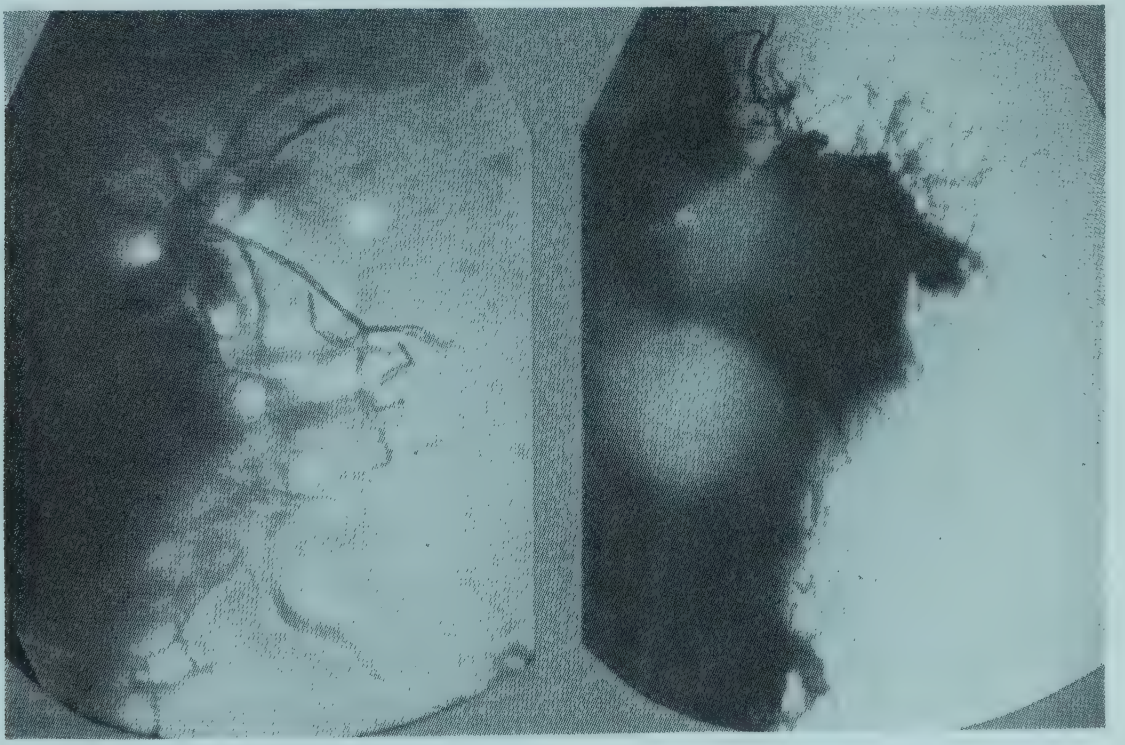

FIGURE 6. Growth of actinomycetes in soil, as shown by direct examination.

where they may exceed in numbers other groups of microorganisms; this was explained by their greater resistance to conditions unfavorable to their nutrition. Beijerinck thus laid the groundwork for our present knowledge of the occurrence and physiology of actinomycetes. He also believed that these organisms play an important role in the humification of the organic matter in soil.

Among the other fundamental studies on the actinomycetes carried out during the early days of this period, mention should be made of the work of Hiltner and Störmer, who made the first comprehensive study of the abundance of actinomycetes in the soil. They found that the season of year and soil treatment have a great influence upon the numbers of these organisms. This work was soon followed by that of numerous other investigators, notably that of Fisher (1909), Fousek (1913), H. J. Conn (1913–1918), Krainsky (1914), and Waksman and Curtis (1916, 1918). The role of actinomycetes in the breakdown of organic residues in the soil, methods for determining their presence and abundance in the soil, and the recognition of the presence of numerous types of actinomycetes received considerable attention.

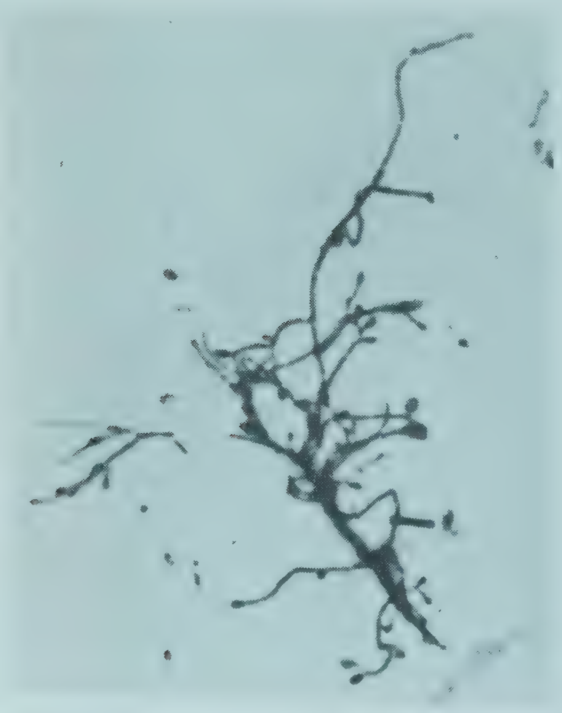

Figure 7. Growth of an actinomycete in a compost, as shown by contact slide method.

Highly significant in this connection was the contribution of Krainsky. His paper published in 1914 may be considered as a classic on a par with the contributions of Cohn, Harz, Thaxter, and Beijerinck. It opened new pathways in the development of our knowledge of the actinomycetes. Krainsky emphasized that the voluminous literature of the actinomycetes contains little of a physiological nature; the numerous descriptions of different species are so much alike that one gains the impression that there are no proper characteristics for distinguishing different kinds of actinomycetes. He emphasized that the terminology as well had not been sufficiently established. Numerous actinomycetes have been isolated, without recognition of their role in nature, since they were always considered from the point of view of their pathogenicity and hygienic importance.

Krainsky's significant contribution was his emphasis of the importance of recognizing the growth characteristics of actinomycetes on synthetic media. He demonstrated that the nature and concentration of both the carbon and nitrogen sources are of great importance in this connection. The nature of the aerial mycelium, pigmentation of the colony, and the formation of soluble pigment are all controlled by the composition of the medium. He further demonstrated that the formation of chromogenic pigments on organic media (tyrosinase reaction) is characteristic not of one but of several species. The formation of invertase and diastase was also found to be characteristic of different species.

Krainsky further established the ability of certain actinomycetes to decompose proteins and cellulose and to reduce nitrate and even suggested the possibility of their being able to utilize the resistant lignin. As a result of these studies he came to the conclusion that the actinomycetes are represented in nature by many distinct species. He suggested that the size and color of colony and the color of the aerial mycelium, under different conditions of nutrition, are sufficient to characterize a species. Physiological properties can be used to supplement such characterization.

Krainsky thus introduced a new approach for characterizing individual species of actinomycetes. Many new organisms were now recognized, and the great abundance of many species in the soil was thus established. Simultaneously and independently, Waksman and Curtis began their work in 1915. Their first paper, published early in 1916, treated the use of synthetic media for characterizing actinomycetes. Before completing their studies, however, they became familiar with Krainsky's work and took full advantage of it in describing various new species. Unfortunately, because of the prevailing World War I, they could not obtain Krainsky's cultures and had to depend solely on descriptions for identification purposes. This led to a degree of confusion in the identification of some of the species. Incidentally, Krainsky's cultures appear to have been destroyed before anyone else had access to them. The work of Waksman and Curtis culminated in a comprehensive study of the cultural properties of actinomycetes by Waksman in 1919.

Attention should also be directed, at this point, to the work of Drechsler, on the morphology of the actinomycetes. Unfortunately, Drechsler made no attempt to isolate fresh cultures from soil or other natural materials, but based all his studies on the cultures submitted to him by Waksman and Curtis. These were all aerial mycelium-producing forms, or species now recognized as members of the genus *Streptomyces*.

3. *Biological Period (1919–1940)*

During this period, not only the morphological and ecological properties of the actinomycetes were studied, but also their physiological and biochemical activities. The publication in 1921, of Lieske's monograph on the "Morphologie und Biologie der Strahlenpilze" may be considered as the beginning of this period. Some of the most outstanding contributions to our knowledge of the taxonomy and classification of actinomycetes, their occurrence in nature, and their antagonistic properties were recognized during this period. Lieske's work, unfortunately, did not exert so great an influence upon the subsequent developments in the field as it should have. This was due primarily to the fact that Lieske was not familiar with the importance of synthetic media in characterizing actinomycetes, nor did he appreciate the fact that these organisms represent numerous species widely distributed in nature. This led him to doubt the existence of more than a few species, which thus tended to obscure rather than to stimulate further developments in this field.

The work of Ørskov, in 1923, on the morphology of the actinomycetes, was highly significant and stimulating, and marked a turning point in the field. It pointed a way toward establishing a new system of classification of actinomycetes, based on their morphological properties. The subsequent investigations of H. L. Jensen, beginning in 1930, on growth of actinomycetes in soil, their morphological and cultural characteristics, tended to broaden further our knowledge of these organisms. In 1934, Duché published a comprehensive monograph on the actinomycetes, made up of species considered to belong largely to the group *Actinomyces albus*. This contribution did not tend to elucidate the nature and activities of the actinomycetes as a whole, but it emphasized the marked variability of their growth on different media and the great complexity of the problem involved. This work was soon followed by the studies of Erikson (1935, 1940) on the pathogenic actinomycetes belonging to aerobic and anaerobic groups and of Kriss (1937) on the variations of actinomycetes.

Considerable emphasis was also laid, dur-

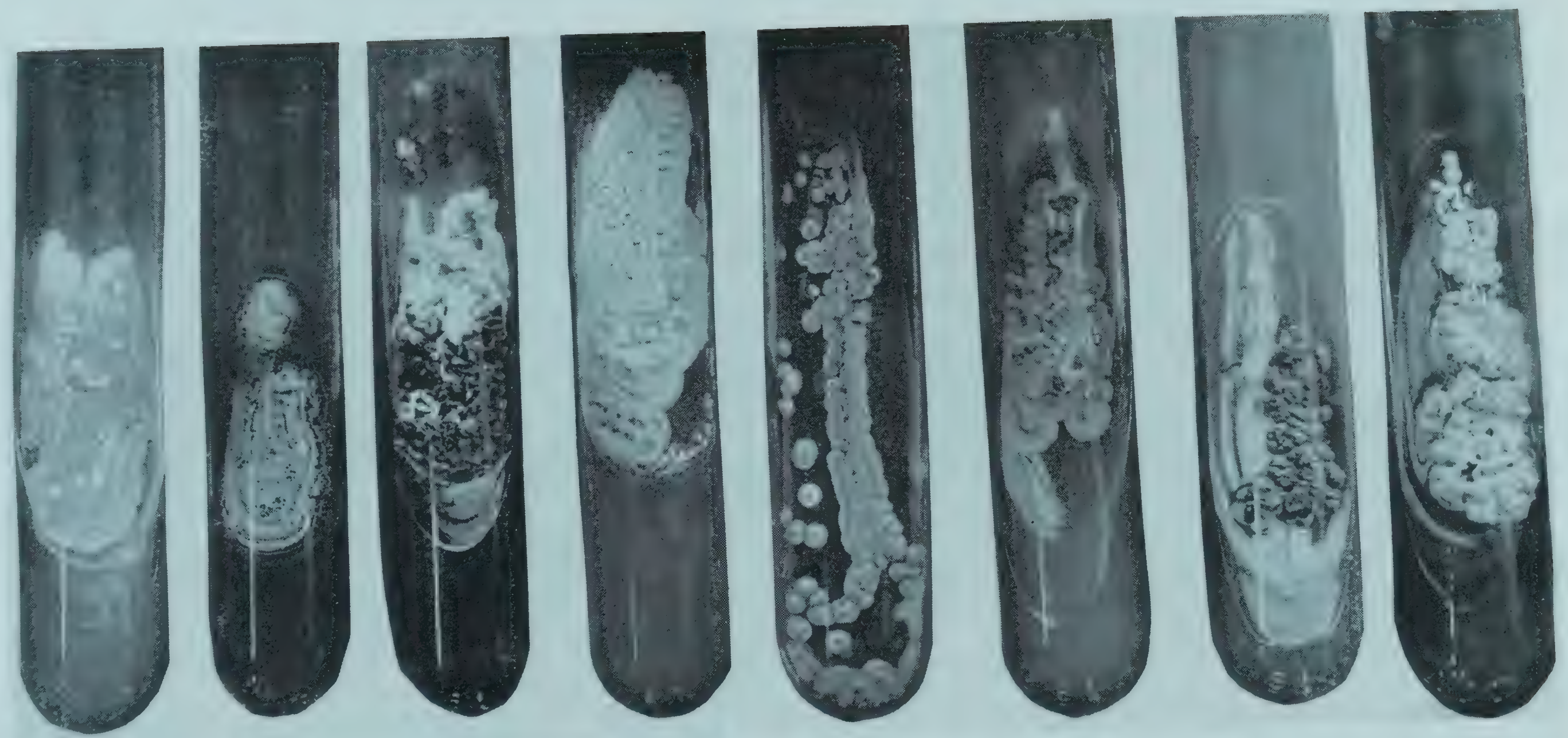

FIGURE 8. A group of cultures of actinomycetes isolated from soil; they represent the genus *Streptomyces* (Waksman, S. A. and Curtis, R. E., Soil Sci. 1: 99–134, 1916).

ing this period, on the metabolism of the actinomycetes, beginning with the work of Waksman and his associates in 1919 to 1924, and leading to numerous other investigations in this field. Studies soon followed on the antagonistic activities of actinomycetes, notably the work of Borodulina (1935), Nakhimovskaia (1937), Waksman and Foster (1938), Alexopoulos *et al.* (1938), Krassilnikov and Koreniako (1939), and numerous others.

The work of Gratia and his group on the formation of bacteriolytic substances by certain actinomycetes was followed by that of Welsch on actinomycetin, a preparation containing an enzyme largely responsible for this activity. The studies of actinophage by actinomycetes were started with the work of Wiebols and Wieringa.

The decomposition of organic materials, ranging from complex substances in the form of plant and animal residues in soil and in composts to pure chemical compounds, including celluloses, hemicelluloses, proteins, and amino acids, received considerable attention, particularly by the writer and his students (Starkey and others).

Information was also gradually accumulating on the systematic position and importance of actinomycetes in natural processes. Several efforts were made to develop a system of classification of actinomycetes that would take into consideration much of the accumulated information. The earlier investigations on the cultural properties of actinomycetes, followed by the studies on their morphology, and their aerobic and anaerobic modes of life, led to certain important systems of classification. The classification of actinomycetes in the various editions of *Bergey's Manual* was based largely upon cultural, biochemical, and certain morphological properties of the actinomycetes. It eventually resulted in a working system of classification, first proposed in 1940, and finally codified (1943) by Waksman and Henrici.

The biological period was also characterized by important contributions to the knowledge of pathogenic actinomycetes, notably the work of Erikson, von Magnus, Naeslund, Cope, Rosebury, and others. The strictly parasitic nature of the facultative anaerobic *Actinomyces bovis* and some of the

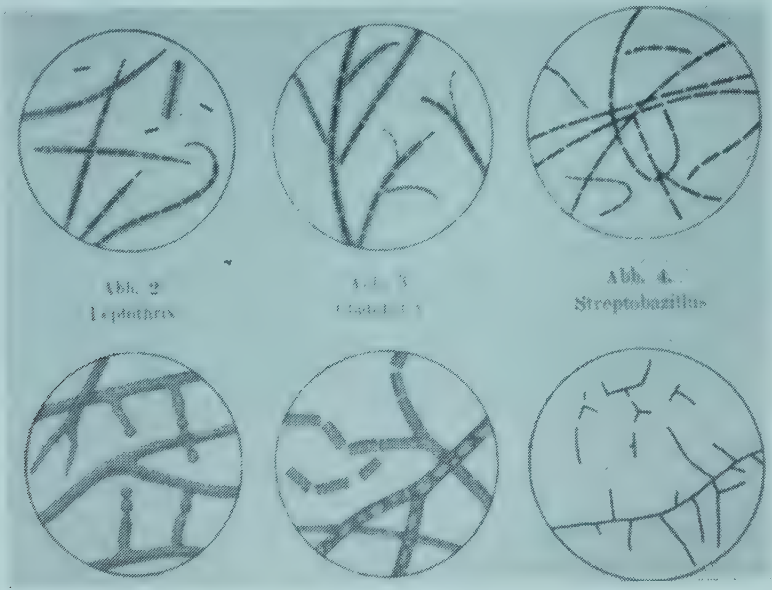

FIGURE 9. Morphology of actinomycetes compared to certain bacteria and fungi with which they have often been confused; l. to r., starting at top: *Leptothrix*, *Cladothrix*, *Streptobacillus*, *Oospora*, *Oidium*, *Actinomyces* (Reproduced from: Lieske, R. *Morphologie und Biologie der Strahlenpilze.* Verlag von Gebrüder Borntraeger, Leipzig, 1921, p. 6).

aerobic *Nocardia* species was now becoming definitely established.

Numerous investigations were made, during this period, on the actinomycetes concerned with the causation of plant diseases. It is sufficient to mention the work of Millard and Burr, Lutman *et al.*, Schaal, and Goss. The organisms concerned in the causation of potato scab were found to belong to the genus *Streptomyces*. The soil and the fresh-water actinomycetes, belonging largely to the genera *Streptomyces* and *Micromonospora*, were also studied extensively. That the importance of actinomycetes as causative agents in human diseases was not neglected is shown by the publications of Colebrook, Henrici (1930), and numerous others. With the introduction of the sulfa drugs as antibacterial therapeutic agents, attention was also directed toward the chemotherapy of diseases caused by actinomycetes.

4. *The Biochemical or the Antibiotic Period (1940–1958)*

The most recent period in the history of actinomycetes has been characterized by a unique development, that of formation and utilization of antibiotics and of certain other biochemical products, notably vitamins. Since the introduction of penicillin as a chemotherapeutic agent, no other group of microorganisms have contributed so much to the field of human and animal therapy as the actinomycetes. Beginning with actinomycin announced in 1940, a large number of chemical compounds have been isolated from cultures of these organisms. Screening programs, initiated in the previous period by a group of Russian investigators and developed by the writer and his associates (1942), found extensive applications.

Considerable attention has also been paid, during this period, to the general problems bearing upon the physiology and biochemical activities of the actinomycetes, especially metabolic processes, enzymatic systems, and biogenesis of antibiotics. Many new species have been described, and a variety of different metabolic reactions investigated. Since this field is so new and since the work done in this connection is so extensive, it is difficult to summarize or to classify the numerous contributions in any sort of chronological order. It is much simpler to divide the period on the basis of certain antibiotic groups that have received the greatest consideration or application. These contributions need not correspond to any particular date or laboratory, since frequently the same active substance has been isolated almost simultaneously in several different laboratories in different countries of the world.

The writer has contributed a number of reviews dealing with the historical background of the development of our knowledge of the antagonistic effects of microorganisms and the production of antibiotics. The first review was published in 1937; it was largely concerned with the antagonistic activities of the organisms themselves, especially with organic matter decomposition and their possible role in soil processes. The second and third reviews were published in 1940 and 1941, and were more concerned with the potentialities of the antagonistic microorganisms as producers of antibiotics. This was followed (1945, 1947) by a comprehensive survey of organisms and their antibiotics in a volume entitled *Microbial Antagonisms and Antibiotic Substances*. Later another volume was published in collaboration with Lechevalier on *Actinomycetes and Their Antibiotics* (1953).

Numerous other reviews and volumes were published dealing with antibiotics as a whole or with certain chemical forms in particular. Only certain of these need be mentioned here: 1, the monumental volumes on *Antibiotics* by H. W. Florey, E. Chain, and their associates; 2, the volume *Actinomycetes Antagonists and Antibiotic Substances* by Krassilnikov; 3, the general reviews by

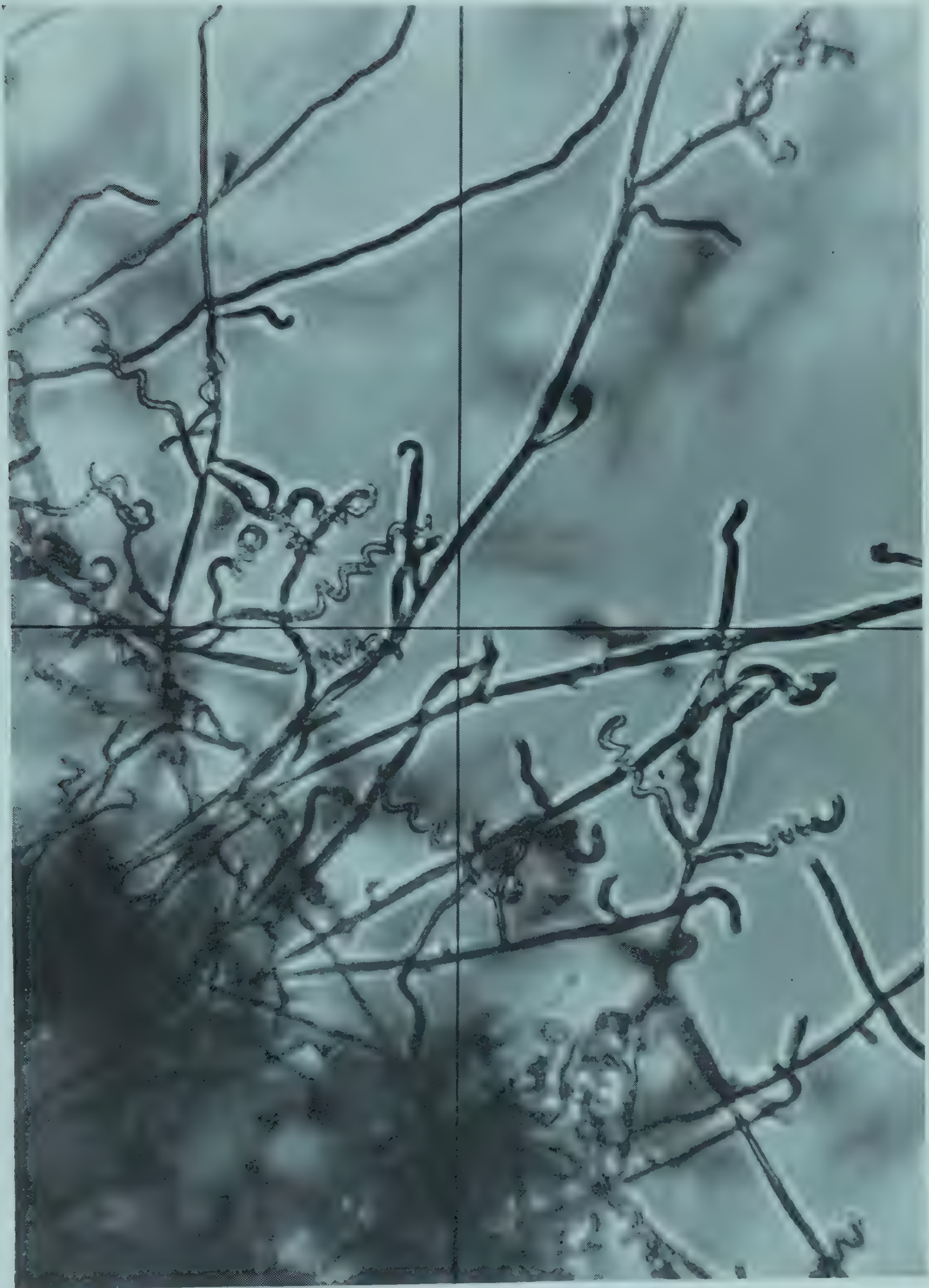

FIGURE 10. The growth of an actinomycete belonging to the genus *Streptomyces*, in artificial culture (Prepared by Grundy of Abbott & Co.).

Benedict and Perlman; 4, finally, the various special reviews of particular antibiotics, as streptomycin, neomycin, the tetracyclines, chloramphenicol, erythromycin, and others.

In addition to the purely antibiotic studies, the period has witnessed great interest in the actinomycetes as a whole, their ecology and classification, morphology and cytology, genetics, biochemical activities and role in natural processes. The numerous journals and volumes devoted to antibiotics contain much material of importance in the study of actinomycetes. This is true of the *Journal of Antibiotics,* published in Japan; *Antibiotiki,* published in the Soviet Union; the annual conference and reports on antibiotics in Washington, U. S. A.; biennial conferences and reports in Moscow, USSR; and in Warsaw, Poland; Italian (Rome, 1953; Milan, 1956) and other conferences and congresses (Vienna, 1958).

CHAPTER 2

Isolation, Identification, Cultivation, and Preservation

Most of the techniques used in the isolation and cultivation of bacteria and fungi also apply to actinomycetes. The isolation of these organisms from soils and other natural substrates is brought about by first plating out such materials in proper dilutions on suitable agar or gelatin media. The plates are incubated at favorable temperaures, for 2 to 7 days, and the colonies* picked and transferred to sterile liquid or solid media for further development. When the culture is found to be contaminated with other organisms, a second plating may have to be resorted to, to obtain pure cultures.

Actinomycete colonies can easily be distinguished on the plate from those of fungi, on the one hand, and of true bacteria, on the other. They are compact, often leathery, giving a conical appearance, and have a dry surface. They are often covered with aerial mycelium. If the colony is well developed and the aerial mycelium abundant, the surface spores can easily be picked with a sterile needle. If growth is limited, or the aerial mycelium not fully developed, sharp, razor-like needles are required to transfer a part of the growth to fresh media.

When grown in liquid culture, either in a stationary or in a submerged condition, the majority of actinomycetes, notably members of the genera *Streptomyces* and *Micromonospora*, grow in the form of flakes or spherical compact masses, leaving the medium clear. The mass of growth can easily be removed by filtration through ordinary paper. Only when growth undergoes lysis do the cells disintegrate completely and a certain degree of turbidity occurs.

Method of Study

In view of the ability of actinomycetes to produce both substrate or vegetative growth and aerial mycelium that usually forms special reproductive cells, known as spores or conidia, it is natural that these organisms should be found in nature both in the form of hyphae or masses of more complex mycelium and as spores. The presence of hyphae or mycelium in the soil or in other materials is usually considered as evidence that actinomycetes lead in such substrates an autochthonous or natural existence and that they form a part of the native microbial population. The presence of spores alone, without mycelium, may suggest that actinomycetes have been introduced there by air or by water.

The methods of studying the actinomycete population of soil, water, compost, and other materials are similar to those used in the study of most of the bacteria and fungi. These methods include: (a) microscopic ob-

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* A colony of an actinomycete is different from a bacterial colony. It represents a filamentous extension of the original cell or cells, spores, and degradation products; it is not an accumulation of cells originating from one or more similar cells.

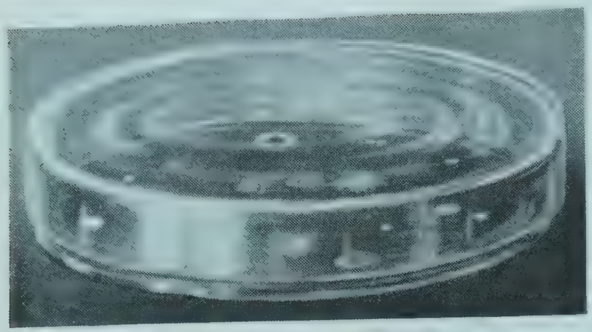


FIGURE 11. Colonies of bacteria and fungi. This early photograph clearly illustrates typical zones of inhibition by some organisms (Reproduced from: Löhnis, F. and Fred, E. B. *Textbook of agricultural bacteriology*. McGraw-Hill Book Co., New York, 1923, p. 28).

servations; (b) plate culture studies; (c) selective culture procedures. Each of these methods has certain advantages and disadvantages, and each contributes toward the elucidation of the nature and abundance of actinomycetes in a natural environment.

Need for Special Media and Standard Conditions of Growth

The problem of pure culture studies of actinomycetes attracted attention in the early days (Bywid, 1889). Great progress has been made since then. At present the culture media used for the growth of actinomycetes can be divided into three distinct categories:

1. Media used primarily for characterization and identification purposes; standard media, comprising both synthetic and organic, are most essential. Synthetic, chiefly inorganic, media have found extensive application in the study of the morphology, physiology, and cultural characterization of these organisms. Organic media are used for obtaining supplementary evidence of a cultural nature, especially for strains that do not grow at all or grow only very weakly on the common inorganic media.

2. Media used primarily for obtaining maximum growth, especially for the maximum production of certain chemical substances, such as antibiotics, vitamins, or enzymes. These are usually complex in composition, utilizing plant and animal materials directly or after preliminary enzymatic or acid digestion.

3. Media used for maintaining cultures of actinomycetes in such a manner as to reduce, to a minimum, degeneration and variation of the culture. Suitable media, comprising both artificial and natural, such as sterile soil, and suitable conditions of growth thus make possible the preservation of type cultures for comparative purposes.

Küster and Grein (1955) made a comparative study of media for the preparation of actinomycete cultures for morphological and physiological studies. They found, for example, that oatmeal agar and potato agar produce exactly the same types of spores, as detected by the electron microscope. Both substrate and aerial mycelium were quite alike on the two media, except for minor differences in the length and degree of curvature of the aerial hyphae. Although the previous cultivation of the organisms frequently affects greatly the physiological constancy of the strains, it did not appear to be the case with the above two complex media.

The great majority of actinomycetes are aerobic; very few are anaerobic; many are microaerophilic. To supply proper aeration, the organisms are grown on the surface of solid media, or in shallow liquid layers, or in a thoroughly aerated submerged condition.

For anaerobic growth, special procedures are required. Temperatures of 25–30° C are usually used for incubation of the great majority of steptomyces, nocardias, and micromonosporas. Pathogenic organisms require 37° C, and thermophiles usually require 50–60° C.

Morphological and Physiological Properties

Among the stable morphological properties of actinomycetes essential for purposes

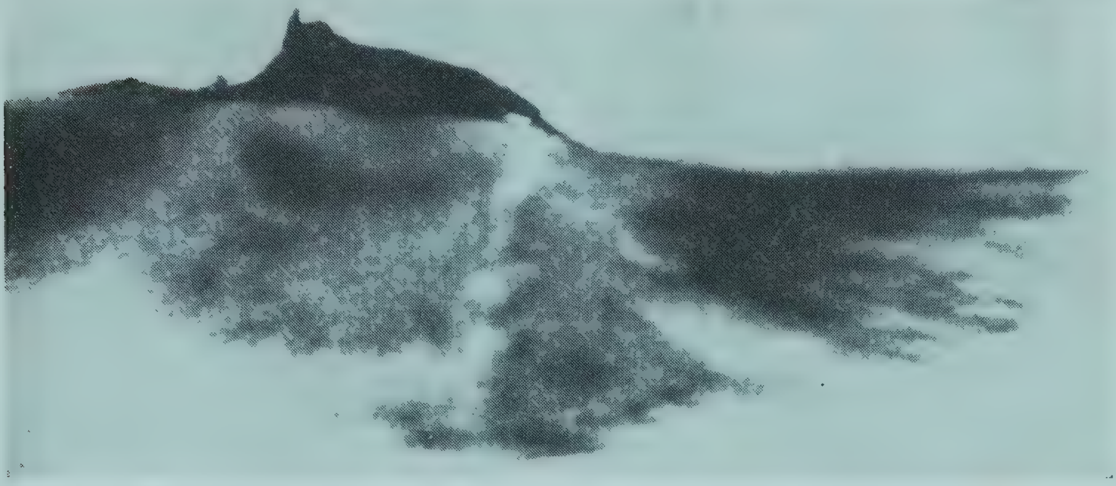


FIGURE 12. Cross-section of a typical actinomycete colony on an agar medium

of characterization and classification, one must list the structure and subsequent changes in the substrate or vegetative mycelium, the production and nature of the aerial mycelium, the nature of the sporulating branches or sporophores, and the size, shape, and surface of the spores.

Among the physiological and cultural properties essential for characterization of actinomycetes, pigmentation of the substrate growth and of the aerial mycelium is most important; the formation of soluble pigments, both in synthetic and in organic media, is also significant. Among the other properties that make possible proper species identification of the organisms are hydrolysis of proteins including gelatin and milk casein, hydrolysis of starch, inversion of sucrose, digestion of cellulose, and formation of specific antibiotics. The utilization of sugars and related compounds, with and without the formation of acids, can supply additional information for species differentiation. The antagonistic activities and the ability to produce antibiotics have recently come into popular use for the characterization of specific organisms. The sensitivity of actinomycetes to specific phages and to known antibiotics is also of considerable importance in establishing specific differences. Among the other supplementary character-

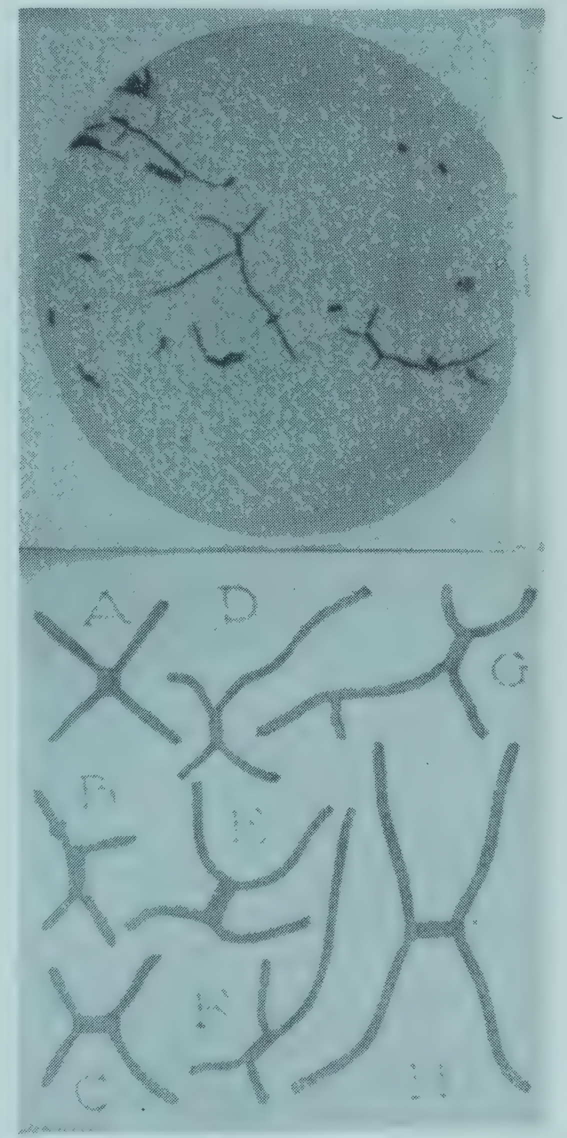


FIGURE 13. Germination of actinomycete spores (Reproduced from: Lieske, R. *Morphologie und Biologie der Strahlenpilze.* Verlag von Gebrüder Borntraeger, Leipzig, 1921, p. 86, 87).

TABLE 2

Classification of streptomyces on the basis of carbon utilization (after Pridham and Gottlieb)

	L-Rhamnose − Raffinose − *S. griseus* *S. lavendulae*	L-Rhamnose + Raffinose + 8-80 group *S. flavovirens* *S. gardneri* A-105	L-Rhamnose + Raffinose − 8-44 *S. antibioticus*
L-Xylose − D-Mannitol − Lactose − Na-acetate (−) (*S. lavendulae*)	L-Xylose + D-Mannitol + Lactose + Na-acetate + (*S. griseus*)		
Dulcitol − DL-Inositol + 8-80 group *S. flavovirens*	Dulcitol + DL-Inositol + (A-105)	Dulcitol − DL-Inositol − (*S. gardneri*)	
Na-acetate + Na-succinate − (8-80 group)	Na-acetate − Na-succinate + (*S. flavovirens*)		
		D-Mannitol + DL-Inositol + (*S. antibioticus*)	D-Mannitol − DL-Inositol − (8-44)

istics, one may list reduction of nitrate and the utilization of fats, paraffin, and phenol.

Pridham and Gottlieb suggested that the utilization of carbon compounds should receive greater consideration for species determination than hitherto. All the particular species tested were able to utilize *d*-glucose, *d*-mannose, dextrin, and glycerol but not erythritol, phenol, cresols, and the sodium salts of formic, oxalic, and tartaric acids. The utilization of such compounds, however, as rhamnose, raffinose, xylose, lactose, mannitol, dulcitol, inositol, and the sodium salts of acetic and succinic acids was selective (Table 2).

Many species of actinomycetes have been described in the literature as pathogenic to both animals and plants. The fact that such cultures were isolated from infectious conditions is not always proof, however, that they are the causative agents of such diseases.

Microscopic Methods

Microscopic studies involve, (a) direct examination of growing cultures; (b) study of hanging drop preparations; (c) use of contact slides, stained and unstained; (d) electron microscope studies; (e) special methods.

Direct microscopic examination. In order to examine the soil, compost, or water microscopically, a dilute suspension of the material is spread over a clean slide, dried, and stained with an acid dye, such as erythrosin

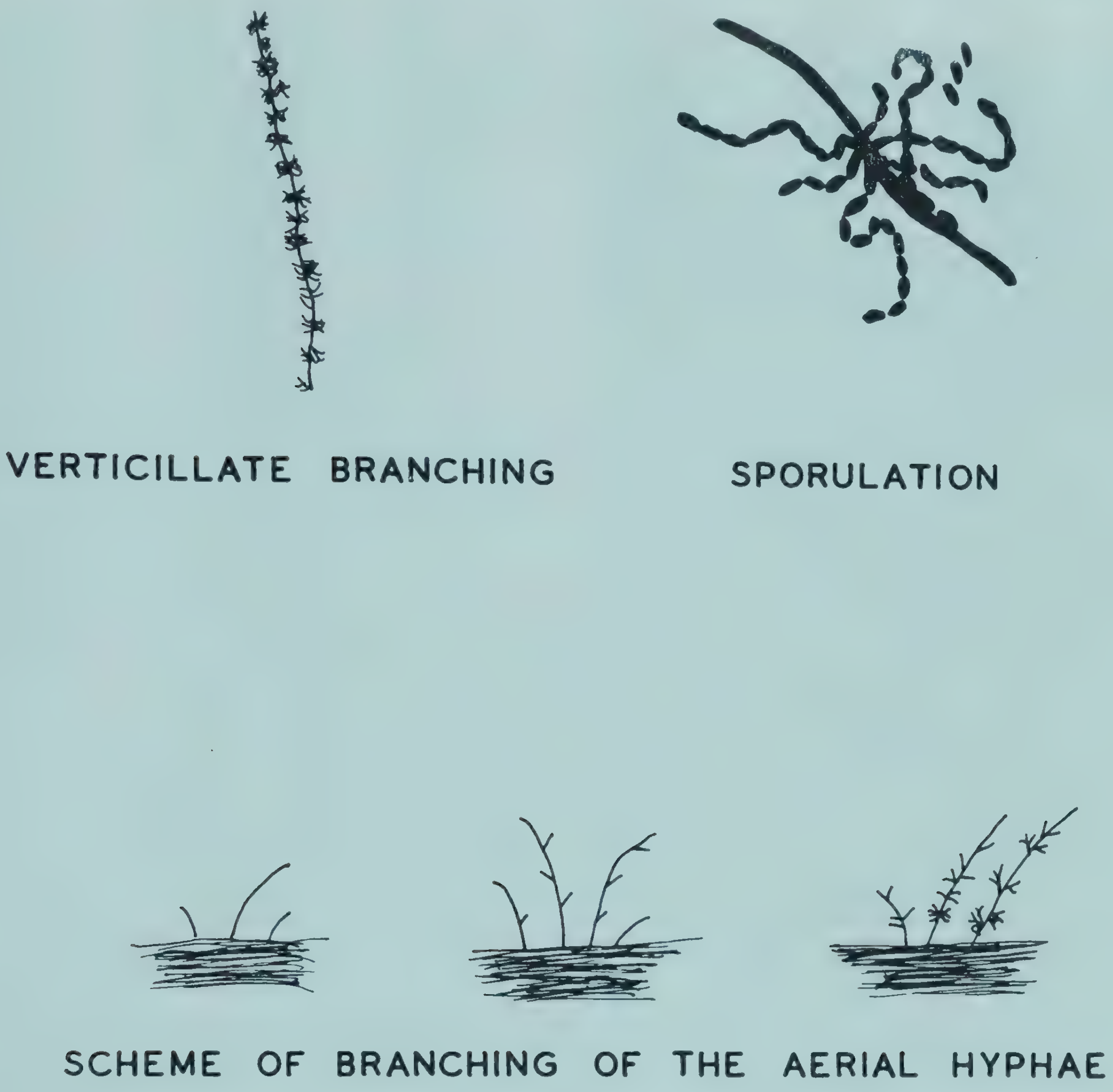

VERTICILLATE BRANCHING
SPORULATION
SCHEME OF BRANCHING OF THE AERIAL HYPHAE

FIGURE 14. A typical verticil forming culture of a streptomyces, *S. verticillatus* (Reproduced from: Kriss, A. E., Mikrobiologiya 7: 107, 1938.)

or rose bengal. Conn found a much greater number of organisms in the soil by this method than could be measured by the ordinary plate methods. By the use of direct staining, actinomycete mycelium was found in abundance in soils, especially those rich in organic matter and not too acid in reaction. This mycelium was not uniformly distributed throughout the soil mass, hence the chief limitation of the method consists in not permitting an accurate quantitative evaluation of its abundance. The fact that the method does not permit the recognition of individual species or even genera is another limitation. The direct examination of undisturbed material offers a distinct advantage, however, which consists in presenting a clear picture of the relative mass of growth of actinomycetes under the particular conditions and in particular substrates.

Kubiena and Renn used a vertically illuminated microscope to examine the soil in an undisturbed state. Actinomycetes were found growing in the soil spaces; aerial tufts of hyphae, in the form of compact colonies with long twisted strands, bridged the soil crumbs. When the soil was enriched with organic materials, the growth of actinomycetes was found to be greatly stimulated.

Contact slide method. This method lends itself quite readily to the study of the actinomycete population of soils and composts. It was first introduced by Rossi and Cholodny. A slit is made with a sharp knife in

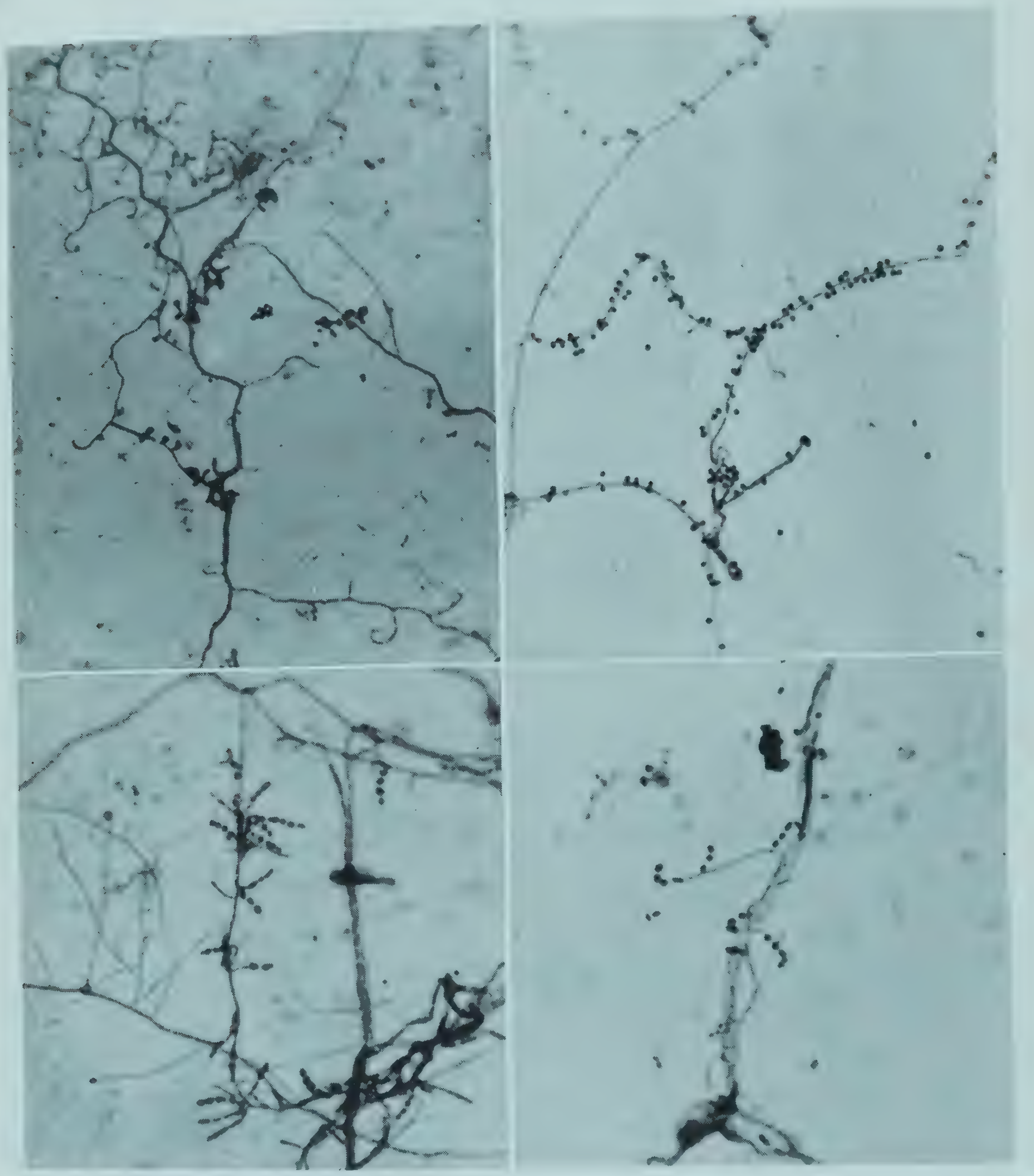


FIGURE 15. Growth of actinomycetes in natural substrates, as shown by contact slide method. Some definitely belong to the *Micromonospora* and others to the *Streptomyces* types.

the material to be examined. A clean cover slide is placed in close contact with the fresh soil, manure, or compost material. The slide is allowed to remain undisturbed for 1 to 3 weeks, then removed, and cleaned on one side. It is dried, fixed over a flame, carefully washed to remove coarse particles of soil or organic residues, and stained with a suitable dye, such as phenol-erythrosin.

The contact slide method offers certain advantages over the direct examination of soil or other material and over the direct staining of such material. It permits the development, on the slide, of organisms present in the substrate and the formation of sporulating bodies. This makes it possible not only to demonstrate the actual presence of such organisms, but also to differentiate and recognize certain broad morphological groups. It is also possible to use this method for the study of the effects of various treatments, such as the additions of plant and animal residues and lime to the soil, response of actinomycetes to different methods of fertilization and cropping, and the relation between saprophytic and plant parasitic forms to the root systems of plants.

Cholodny observed that the direct microscopic method does not give so accurate a picture of the abundance of actinomycetes in the soil as does the contact slide method. The latter, however, has one distinct disadvantage, since it does not permit estimation of the relative abundance of actinomycetes in the soil or in other natural material, such as foodstuffs, manure, or water.

Conn observed that an increase in the moisture content of the soil favored a change in the microbial population from that of actinomycetes and fungi to bacteria. Waksman, Umbreit, and Cordon were able to record the change in the actinomycete population of composts by the use of the contact slide. Numerous forms were detected that could not be found readily by other methods.

For the microscopic examination of colonies of actinomycetes, Nishimura and Tawara used the agar-cylinder method. This consists in pouring 25 ml of nutrient agar into a sterile Petri dish and allowing it to harden. A previously sterilized cork borer, about 8 mm in diameter, is plunged through the agar at several sites on the plate. Each agar cylinder is dug out carefully with a sterile hooked wire needle. A loopful of spore suspension of the culture is placed on the surface of each agar cylinder. With the aid of a flame-sterilized pincette, a previously sterilized clean coverglass is pressed down over each cylinder so that the corners of the coverglass are equidistant from the center of the agar cylinder. The petri dish is covered and incubated at 28° C. When the culture has reached the proper stage of development, one of the coverglasses is removed from the agar cylinder with the sterilized pincette. The growth of the mycelium clings to the undersurface of the coverglass, forming a ring. The microscopic examination of the periphery of this circle makes it possible to observe both aerial and vegetative mycelium, in an undisturbed condition, and also to follow the growth of the organisms at every stage of their development.

Permanent slides are stained and prepared as follows. The ring of the growing mycelium on the undersurface of coverglass is fixed by means of 2 or 3 drops of absolute methanol for 10 minutes. The preparation is then washed with tap water and dried before it is stained. Among the stains tested, the following gave the best result: Giemsa solution, crystal violet, eosin, methyl violet, hematoxylin, methylene blue, and carbol fuchsin, the first two stains allowing a clear picture of the sporulation process. Numerous modifications of the staining of actinomycete colonies and their previous cultivations on specially prepared media or under special conditions have been described.

Among the various modifications of the methods of examination of colonies of actinomycetes, the cellophane procedure deserves further consideration. Erikson (1940) grew cultures of actinomycetes on sterile cellophane placed on agar of different compositions. Portions of the growth were removed at different intervals. Both the substrate and aerial mycelium could thus be stained and examined. The cellophane bearing the growth is stained for 30 minutes in a 70 per cent butanol solution of Sudan IV, dipped in 70 per cent ethanol, washed in water, and mounted on slides. This method was modified by Giolitti and Bertani and others. Excellent differentiation can thus be obtained for the vegetative and sporulating growth.

Electron microscope methods. The electron microscope has opened a new field for the detailed study of the finer structure of cells of actinomycetes. References to the results thus obtained are presented in Chapters 5 and 6, and elsewhere throughout this volume.

Plate Methods

The plate methods are the oldest and still most reliable procedures for determining the nature and measuring the abundance of the microbiological, including the actinomycete, population of such materials as soils, composts, water, and milk. In making microbiological counts of such materials, the actinomycetes are usually included with the bacteria. More recently, selective media favoring the preferential development of actinomycetes have been devised, as by addition of sodium propionate to the plate (Crook *et al.*, 1950) or by addition of antibiotics. Certain purification procedures, such as the use of various concentrations of sulfuric acid, have also been utilized (Fellinger, 1931).

Beginning with the work of Hiltner and Störmer in 1902, actinomycetes came to be recognized as a separate group of organisms, distinct from the bacteria and fungi, and were frequently so reported. The samples of soil, compost, or other material are usually suspended in sterile tap water, and various dilutions with sterile tap water subsequently made. These dilutions are then plated out on suitable agar or gelatin media. The plates are usually incubated at 28–30° C and examined after 2 to 7 days. It is sometimes difficult to differentiate between bacterial and actinomycete colonies, unless one has had considerable experience with actinomycetes or unless one uses microscopic magnifications to distinguish between the two types of colonies.

At first, organic media were used to enumerate the abundance of actinomycetes in the soil. More recently, synthetic media have been employed with only small amounts of a protein or polypeptide added, such as 0.005 per cent peptone, 0.025 per cent powdered egg albumin, or 0.02 per cent casein. This tends to prevent development of the more rapidly growing and spreading bacterial and fungal colonies and allow development of the more slowly growing actinomycetes. The various water dilutions are so adjusted as to allow a final count of only about 30 to 100 colonies per ordinary Petri plate.

If one is interested in obtaining a great variety of actinomycetes for isolation purposes, rather than in large numbers of colonies, synthetic media are best for plating purposes. With asparagine, glutamic acid, or sodium nitrate as a source of nitrogen, and glycerol, starch, glucose, or a salt of an organic acid, such as malate, as a source of carbon, a relatively simple medium can be prepared. Such a medium will favor the development of actinomycetes rather than of fungi and bacteria. The colonies of actinomycetes produced on such media are quite characteristic and can easily be recognized.

Numerous modifications of the plate methods have been proposed, depending on such factors as the treatment of the soil, the nature of the medium used, and the temperature and length of the incubation period (Rao and Subrahmanyan, 1929). Some methods are devised for the purpose of excluding most of the bacteria and fungi. In others, an attempt is made to distinguish between the abundance of viable spores and mycelial fragments of the actinomycetes. Skinner made use of the fact that, when shaken in suspension, vegetative mycelium of actinomycetes breaks into viable fragments, which are killed when shaking is prolonged (Table 3). He observed further that spores do not break up into small particles and are more resistant to shaking than are vegetative fragments. Vegetative mycelium may thus be distinguished from spores by the manner in which the viable count varies with time of shaking. Although it was not possible to estimate the numbers of spores and of vegetative particles in mixed suspension, certain predominating forms could be identified.

Selective Culture Methods

The selective culture methods for the isolation of actinomycetes are based on the use of media that contain specific nutrients favorable for selective development of the organisms. Such media may contain nutrients favorable to actinomycetes, because of their specific metabolism, such as paraffin utilization, steroid oxidation, or keratin decomposition. On the other hand, substances are added to the medium which may discourage the growth of fungi and bacteria. With the growing appreciation of the biochemical potentialities of various actinomycetes, especially as regards formation of antibiotics, vitamins, and enzymes, the development of special media for the enumeration and isolation of particular organisms has great possibilities.

The incorporation of antibiotics and other antimicrobial substances in such media offers some very interesting potentialities. On the one hand, certain antibiotics may be used to repress bacteria or fungi and allow only particular actinomycetes to develop. On the other hand, antibiotics may be employed for the selection of vigorous strains of organisms capable of producing these antibiotics.

For the purpose of suppressing fungi, Corke and Chase suggested the use, in plating media for actinomycetes, of cycloheximide (40 μg/ml of medium added just prior to the pouring of the plates). The presence of the antibiotic not only results in the repression of the fungi, but also gives much larger numbers of actinomycetes. This procedure was found to be superior to the use of sodium propionate, mentioned previously (Crook *et al.*). The incorporation of nitrofurazone or other antibacterial agents will also favor the development of actinomycetes on the plate, as shown by Yoshioka. Unfortunately, no antibiotic or other antimicrobial substance has yet been found that would inhibit selectively the separate genera of actinomycetes.

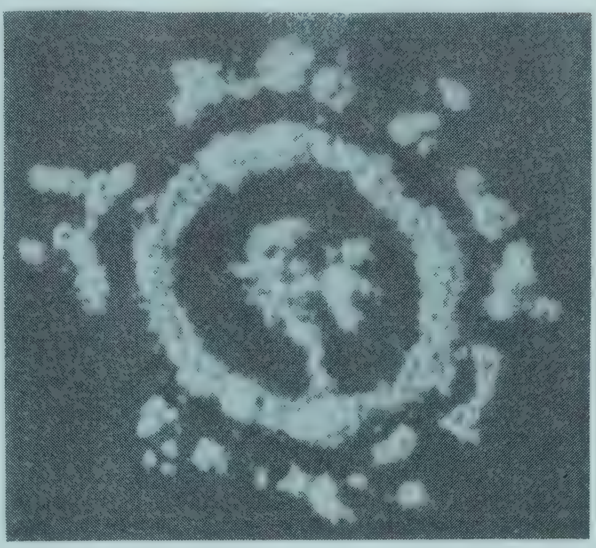


FIGURE 16. Typical zonation in the aerial mycelium of a streptomyces colony (Reproduced from: Lieske, R. *Morphologie und Biologie der Strahlenpilze*. Verlag von Gebrüder Borntraeger, Leipzig, 1921, p. 168).

TABLE 3

Effect of shaking upon suspensions of substrate actinomycete mycelium (Skinner)

Time of shaking	Dilutions and colonies per plate*	
	1:10	1:100
min		
0	2.7	1.3
5	151.0	180.0
15	133.3	126.7
30	69.7	37.0
60	12.7	3.3
Plating dilutions	1:2000	1:200

* Vegetative culture diluted in saline; mean of three replicate plates reported.

The addition of antibiotics to media for the purpose of enriching specific antibiotic strains was first suggested by Waksman *et al.* for obtaining highly potent antibiotic-yielding strains from a particular antibiotic producing organism. It was also used by Umezawa *et al.* (1949) for the isolation of chloramphenicol-producing organisms.

For the isolation and cultivation of members of the genus *Actinomyces*, or the mesophilic anaerobic forms, special methods have to be employed. M. H. Gordon suggested the use of ordinary nutrient broth to which a few drops of fresh human blood have been added. The material is inoculated into two lots of blood broth, one of which is covered by a layer of oil 1 cm deep. After incubation for a few days at 37° C, the organism can be seen growing, at the foot of the tube in small white masses—like little puffballs. According to Gordon, growth occurs first in the broth covered with oil; when other bacteria are also present the actinomyces may appear first in the aerobic tube.

Various other methods have been used for the isolation of microaerophilic strains of *Actinomyces*. The method proposed by Rosebury *et al.* (1944) for the isolation of this organism from gingival scrapings was modified further by Ennever *et al.* (1949). A tooth-bearing removal appliance is used to collect the plaque mass. The latter is removed with a sterile, hooked blade and placed in a micromortar containing 0.05 ml of sterile saline with Triton A-20 at 1:200 dilution. The mass is triturated with a sterile glass pestle, and one visible granule transferred by means of capillary pipette to brain-heart infusion agar. Care is taken to transfer as little saline as possible. The granule is streaked upon the agar, and plates are incubated anaerobically in an atmosphere of 5 per cent CO_2 for 6 days.

Preserving Cultures of Actinomycetes

The great variability of actinomycetes, frequently accompanied by loss of desirable properties, makes it necessary to develop methods for the maintenance of cultures in which the organisms will undergo the least morphological or biochemical change. The ability to form spores is an important desirable property to be conserved.

Various methods are used for the preservation of actinomycete cultures. Artificial media, including both synthetic and organic, have been used for maintaining cultures of actinomycetes. As a rule, organic media have been found more favorable than synthetic media for maintaining the original morphological and cultural properties of actinomycetes.

R. Gordon of the Rutgers Institute of Microbiology has reported her experiences in comparing three methods for the preservation of the culture collection of actinmycetes at the Institute.

1. *Soil culture.* Thirty-nine strains of *Streptomyces* were added to sterile soil. All of them were viable a year after inoculation; 2 years after inoculation three of the 39 did not grow; 3 years after inoculation, six of the 39 did not grow.

2. *Mineral oil.* Twenty-four cultures of *Nocardia* and *Streptomyces* were covered with sterile mineral oil. Three years later, 14 of the cultures were viable and 10 were dead.

3. *Lyophilization.* All strains of actinomycetes in the Institute's collection have been lyophilized, and no difficulty in reviving them has been experienced. In the regular schedule of testing lyophilized cultures for viability, 33 strains of actinomycetes have grown after 5 years' storage; none have failed to grow.

Hartsell reported that the grisein-producing strain of *S. griseus* was viable after 7 years' storage under mineral oil and that the streptomycin-producing strain of this organism was viable after 6 years' storage under the same conditions.

Haynes *et al.* carried out extensive investigations on the preservation of the collection of more than 2800 cultures at the Northern Regional Research Laboratories of the U. S. Department of Agriculture. Lyophilization has been relied upon exclusively, for several years, for the preservation of the bacteria, including the actinomycetes. No actinomycete was encountered which did not survive lyophilization. With few exceptions, the lyophilized cultures have remained viable for as long as they have been under observation, some being 14 years old.

Pridham *et al.* (1956) made a detailed study of the different media favorable to the maintenance of the desirable properties of actinomycetes. A comparison was made of 500 strains grown on 19 different media. Yeast-glucose and tomato-paste oatmeal agar allowed the growth of 85 to 86 per cent of the cultures with the formation of abundant aerial mycelium. Potato-glucose agar, however, gave only 12 per cent viability. Glucose-asparagine agar and synthetic (known as Czapek's) agar were among the worst.

Soil has been found very effective in maintaining actinomycete cultures. Several methods are used in the preparation of the soil cultures. A good, fertile soil is selected and freed from roots and pebbles. One-hundred gm portions of such soil are placed in 250-ml Erlenmeyer flasks. If the soil is acid, 1 gm of ground $CaCO_3$ is added. If the soil is poor in organic matter, 0.25 gm of dried blood or casein is added.

Pridham *et al.* (1956) described the following method. About 2 gm of finely ground, loamy soil is placed in each of a number of 10- by 100-mm tubes. These are plugged with cotton and sterilized four times for 30 minutes at 121° C, on alternate days. At the end of the fourth sterilization period, tubes (selected at random) are tested for sterility by the addition of broth and incubation at room temperature (25 to 30° C) for 1 week. In no instance did growth of microorganisms occur in the soil-broth suspensions. The details of the method were further described as follows:

"Soil cultures were prepared by inoculating 10 ml of broth in a 25- by 150-mm culture tube with the growth from a slant culture of a particular streptomyces. The inoculated tubes were placed on a rotary shaker and incubated with shaking for 2 days at 28 to 30° C. Two ml of the broth culture were pipetted into a tube of sterilized soil and the suspension thoroughly mixed with the tip of the pipette. The tubes were plugged and allowed to air-dry at room temperature for 2 to 3 weeks. During this period, growth usually occurred in the soil-broth mixture, appearing on the surface as a mat that was often covered with abundant aerial mycelium."

Other experiences were reported by Frommer (1956).

Additional Methods and Media

Numerous additional reports are found in the literature on the methods for the cultivation of actinomycetes, and for the study of their morphological and physiological properties. Some of these methods are specialized in nature, as, for example, the methods for the isolation of antibiotic-producing strains of *Streptomyces* (Waksman *et al.*, 1946, 1947), and the principles of the screening program for antibiotic-producing organisms (Waksman and Lechevalier, 1951). Additional studies have been made by Valyi-Nagi and Szabo (1957). The standard media (Waksman, 1950) used in the growth of actinomycetes, both for descriptive purposes, and for morphological and physiological investigations, will be given in Vol. II, Appendix 2. Details of staining procedures of cultures of actinomycetes will be given in Vol. II, Appendix 3.

Tresner and Backus proposed another simple method for the preservation of cultures of actinomycetes. Spores of the organisms are streaked on appropriate agar media in

Petri dishes and incubated for 2 weeks at optimum temperatures. One ml of 40 per cent formaldehyde is then added dropwise to the surface of the agar outside and between the growth zones. The plates are kept at room temperature with covers in place until all of the formaldehyde has been absorbed. After 2 to 3 hours, the plates are sealed with rubber sealers and stored at 4° C. Cultures so treated have retained most of their original characteristics for longer than 2 years. The plates can also be stored at room temperature for several weeks. Morphological features, color of spores or mycelium, and growth habit remained essentially unchanged in most cultures. In a few cases, changes in pigmentation have been observed when the formaldehyde was added.

CHAPTER 3

Distribution in Nature

The distribution in nature of any group of living organisms, whether these are higher or lower forms of life, with or without the power of locomotion at least at one stage of their development, depends on several important factors:

1. The nature of the substrate in or upon which the particular organisms live.
2. The nature of the food supply available to them as sources of energy and for cell synthesis.
3. The nature of the environment, notably aeration, temperature, and reaction.
4. The biotic complex, or the specific nature of other living systems with which the particular organisms have to come in contact, thereby possibly competing for space and nutrients.

The mode of nutrition of these organisms, the synthesis of various chemical complexes, the formation of waste products, and the mechanisms (such as enzyme systems) whereby these reactions are brought about,—all depend, to a large extent, upon the nature of the organisms, the available food supply, and their ability to adapt themselves to the particular environment.

The wide occurrence of actinomycetes was reported by many of the early investigators. Those who did not recognize this fact frequently reported, as the causative agents of a particular infection, air contaminants which they isolated. Others observed the wide occurrence of the actinomycetes but considered the organisms to be only different forms of the same general type. It is sufficient to cite the following.

Salerazés, in 1895, spoke of different kinds of "Streptothrix" capable of producing actinomycosis in man. He believed, however, that actinomycosis could be caused by different other forms, a situation he considered as sufficient explanation for the variations frequently reported in the morphology and biology of *Actinomyces bovis.* He also spoke of the occurrence of "Streptothrix" in air and water. Although he considered them as external saprophytes, living on vegetables and cereals, he believed that they were capable of causing human and animal infections. The mouth was looked upon as the portal of entry of the organisms. He believed that they did not produce toxic secretion products, but caused injury through their actual vegetative development ("vegetabilité") in the body.

Distribution of Actinomycetes

Actinomycetes are widely distributed in nature. They are found in virtually every natural substrate; in the air we breathe, in the water we drink, in the foodstuffs we consume, and in the soil we walk on. Soils and composts are particularly favorable for their development; they are found there in great abundance, both in numbers and in kinds. The deep seas, however, do not offer so favorable a medium for their development. Some substrates are ideal as permanent habitats for the actinomycetes, where they live and multiply; other substrates represent only temporary habitats for actinomycetes, where they are distributed by water and air movements. They are also found in the far north and on high mountains, in deep layers of soil, and in oil deposits. Some genera favor one habitat and others favor another. *Streptomyces* is most commonly represented in soils and in composts; *Micromonospora* species are abundant in lake bottoms; some members of the genera *Actinomyces* and *Nocardia* are known to be causative agents of human and animal diseases; *Thermoactinomyces* and other thermophilic genera grow abundantly in stable manure and in high-temperature composts. Enghusen (1956) explained the wide distribution of *Streptomyces* species by the small size of their spore (1.0–1.5 by 0.5–0.8 μ) and by their resistance to drying (for 3 years or more).

On the basis of their actinomycete populations, from both a quantitative and a qualitative point of view, the following natural substrates may be recognized:

1. Soils, comprising virgin and cultivated, garden, field, and forest soils, as well as drained peat bogs.
2. Sea waters and sea bottoms.
3. Fresh water basins, comprising lake and river waters and bottoms.
4. Manures and composts.
5. The atmosphere.
6. Food products, including milk.
7. The bodies of plants: some find in and upon the plant a temporary or permanent habitat; others are able to cause diseases of plants.
8. The bodies of man and animals, especially the digestive system.
9. Geological formations.

Occurrence and Abundance of Actinomycetes in the Soil

The distribution of actinomycetes in various soil types began to receive attention soon after the recognition of their existence as a separate group of microorganisms. The exact enumeration of the large numbers of species found in the soil was actually begun, however, during the first years of this century.

For a time after the first designation and description of an actinomycete by Cohn, but little attention was paid to the occurrence of actinomycetes in nature, aside from the animal pathogens. Globig was among the first to draw attention, in 1888, to the occurrence of actinomycetes in the soil. He isolated a thermophilic organism, using potato as a medium. Another form was soon isolated from the air by Rossi-Doria (1891) and designated *Streptothrix alba*. In 1900, Beijerinck established that actinomycetes occur in great abundance in the soil. He found them in garden soil at a depth of 1 m, in sandy soil to a depth of 2 m; he also found them in the bottom mud of a river bed. Beijerinck emphasized that actinomycetes are omnivorous organisms, living and growing under a great variety of conditions.

The first quantitative enumeration of actinomycetes in the soil was made by Hiltner and Störmer, in 1903. The gelatin plate method was used. The numbers of actinomycetes were found to vary between 13 and 30 per cent of the total microbial flora of the soil capable of developing on the plate. These variations depended primarily on the season of year; from 20 per cent in the spring, they dropped to 13 per cent in summer, and rose to 30 per cent in the fall. The last increase was ascribed to the addition of fresh undecomposed plant and animal residues. The introduction of stable manure into soil also had a marked effect in increasing the number of actinomycetes.

Fischer found that actinomycetes comprise 15 per cent of the microbial population of sandy soils. Fousek reported that the highest numbers of actinomycetes are found in the fall of the year (27 to 35 per cent of all colonies); the lowest number occurred in

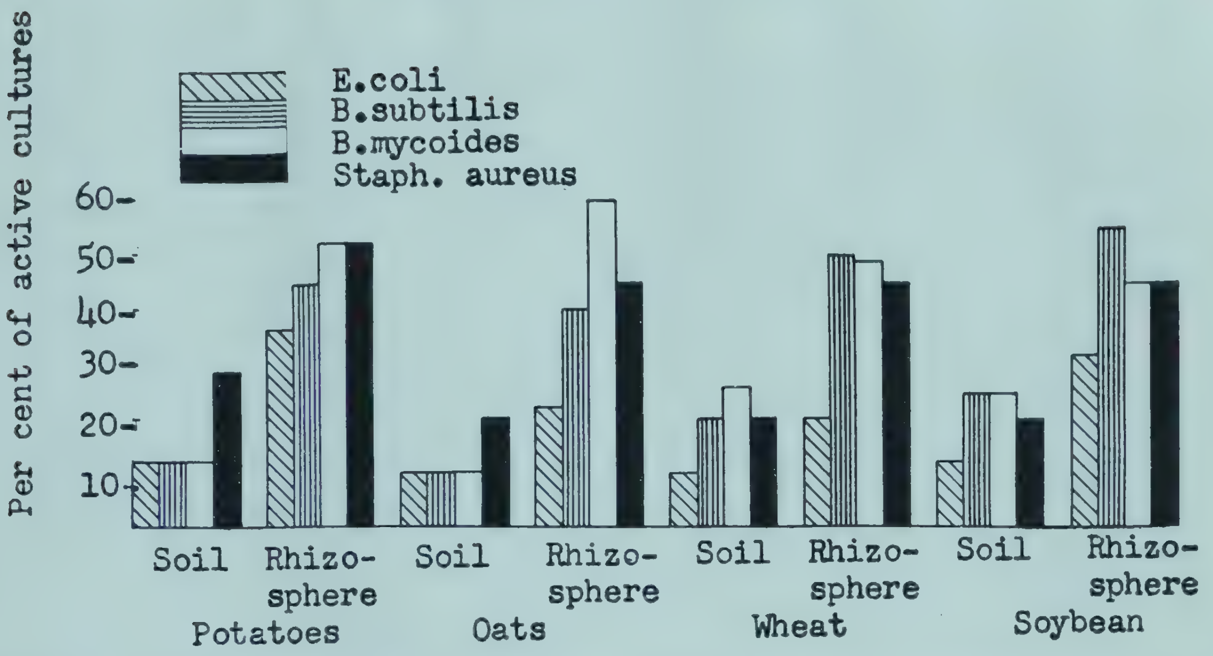


FIGURE 17. Effect of plant roots on the distribution of antagonistic actinomycetes in soil at an early stage of plant growth (Reproduced from: Rouatt, J. W., Lechevalier, M., and Waksman, S. A. Antib. Chemoth. 1: 190, 1951).

the spring (18 to 23 per cent). He also concluded that the addition of fresh organic residues in the fall was largely responsible for the increase in the number of actinomycetes. Their presence in forest soils and on the roots of grasses and leguminous plants was considered as further evidence that they play an important role in the decomposition of plant residues. These observations were confirmed by Conn in 1916, who reported that actinomycetes make up as much as 40 per cent of the microbial population in soils rich in plant roots, as compared to the population of cultivated soils, where the numbers of actinomycetes were only about 21 per cent. Extensive studies on the actinomycete population of the soil were also made by Münter.

Krainsky obtained much lower numbers, however, because the synthetic media he used permitted the growth of only 20,800 actinomycetes per gram of soil. Although valuable for the recognition of a greater variety of species, these special media do not allow the development of so great a number of total colonies of actinomycetes as do organic media.

Waksman and Curtis also reported that soils contain large numbers of actinomycetes. Although the actual numbers diminished with depth of soil, they increased in proportion to the bacteria. At the surface of certain soils, actinomycetes, as measured by the number of colonies produced on agar plates, made up 9 to 15 per cent of the total population, or a total of 743,000 to 933,000 per gram of soil. At a depth of 30 inches, the numbers dropped to 240,000 per gram, but the percentage rose to about 66. In California soils, the numbers varied from 380,000 to 1,890,000 per gram, and the percentage of the total population from 19 to 45.

The total and relative numbers of actinomycetes in a number of American soils, as determined by the use of egg-albumen agar, are shown in Table 4. Acid soils, waterlogged soils, and soils poor in organic matter contained the lowest numbers of actinomycetes. Heavy soils and organic matter-rich soils contained the highest numbers.

Gillespie and Waksman and Joffe found that the critical degree of acidity for the growth of the majority of actinomycetes in the soil is pH 4.8 to 5.0, the optimum reaction being pH 7.0 to 8.0. Jensen (1928) iso-

TABLE 4

Numbers of bacteria and actinomycetes in various soils, as determined by the agar plate method
(Waksman and Curtis)

Nature of soil	Bacteria	Actinomycetes	
	thousands per gm	thousands per gm	per cent of total organisms
New Jersey garden	5,300	900	14.5
New Jersey orchard	4,800	700	13.4
New Jersey meadow	8,100	550	6.3
New Jersey forest	610	110	15.3
Iowa loam	1,764	236	11.8
Cranbery soil	204	7	3.5
Louisiana sandy loam	8,300	1,700	17.0
California fertilized soil	3,570	630	15.0
California unfertilized soil	580	330	36.3
California adobe	3,620	800	22.0
California sandy loam	6,010	1,430	19.2
Oregon adobe	13,100	2,400	15.4
Porto Rico clay loam	2,140	960	31.0
North Dakota wheat soil	2,067	933	31.1
North Dakota flax soil	1,737	263	13.2
Hawaiian pineapple soil	4,334	666	13.3
Alaska soil	6,034	1,566	20.6
Texas Lufin fine sandy loam	2,126	574	21.3
Colorado alfalfa soil	3,440	1,560	39.0
Maine potato soil	4,650	250	5.1
Maine infected soil	15,900	2,200	12.2
Alberta grass soil	1,110	760	40.6
Alberta garden soil	2,000	1,700	46.0

TABLE 5

Numbers of actinomycetes in Indian soils
(Rao and Subrahmanyan)

Description of soil	Crop raised	Actinomycetes, thousands per gm of soil
Black cotton.		3340
Alkaline.	Wheat	2540
Peaty.	Paddy	2340
Alluvial.	Paddy	1000
Reddish laterite.	Tea	250
Kalar soil.	Fruit	680
Red sandy loam.	Coconut	40

lated from the soil a special acidophilic organism. The soil reaction had a marked influence upon the numbers of actinomycetes, although there was no direct correlation between numbers and reaction. He suggested that the low numbers in strongly acid peat soils may be due largely to the poor aeration prevailing in such soils. The addition of lime brought enormous increases in the numbers of actinomycetes. In a study of 56 Danish soils, Jensen reported that the numbers varied from none to about 13 millions per gram, their percentage of the total microflora developing on the plates being 0 to 73.3 per cent. In Australian soils, he (1934) found the numbers of actinomycetes to vary from 10 to 50 per cent of the total microflora.

Using a starch medium with traces of nitrogen and certain minerals, Rao and Subrahmanyan reported the presence in Indian soils of 40,000 to 3,340,000 actinomycetes per gram, as shown in Table 5. In a study of the abundance of actinomycetes in heavy loam Rothamsted soils, Singh observed a broad relation between numbers of these organisms and soil fertility: the heavily manured soils gave the highest numbers and the unmanured the lowest.

Under certain exceptional conditions actinomycetes may make up the predominant part of the soil microbiological population. This was reported, for example, by Johnstone for the soils in the Bikini and Rongelap Islands in the Pacific. The organic matter content of the Bikini soil varied from 1.2 per cent at the surface to 0.56 per cent at a depth of 6 inches, and the pH from 8.7 to 9.2. The total numbers of actinomycetes were low, varying from 10,000 per gram at the surface to 20,000 in the upper 6-inch layer. The corresponding numbers of bacteria were even lower, or about 1,000 to 2,000. The Rongelap soil was less alkaline (pH 8.0 to 8.4) and contained much larger numbers of actinomycetes, ranging from

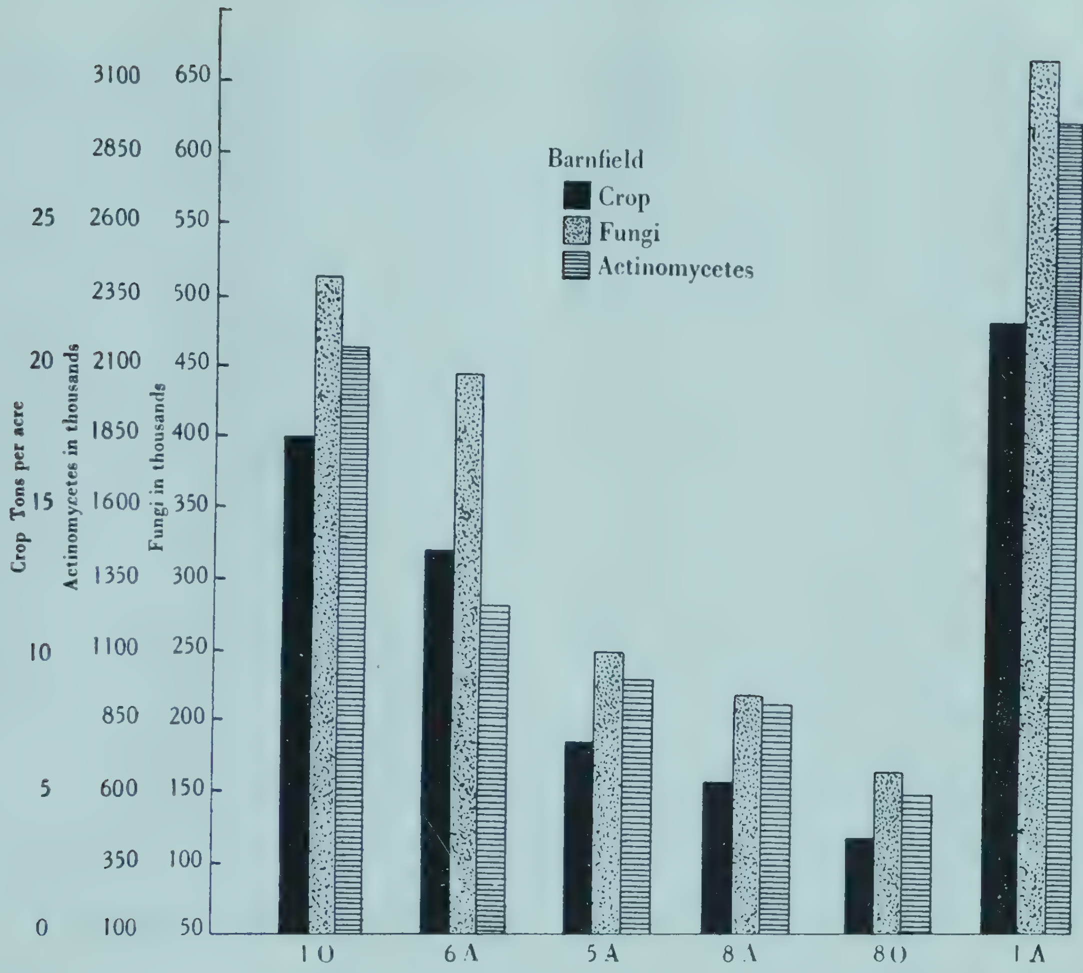


FIGURE 18. Relationship between crop yields and numbers of actinomycetes (Reproduced from: Singh, J. Ann. Appl. Biol. **24:** 163, 1937).

10,000 to 4,000,000 per gram. But here again the bacterial numbers were considerably lower, or 1,000 to 160,000 per gram.

Krassilnikov (1938) recorded much lower numbers of actinomycetes for various Russian soils: they made up only 5 to 7 per cent of the total number of colonies developing on the plate. The soils of the dry steppes of Northern Kasakhstan were characterized (Tepliakova and Maximova) by a significant number of actinomycetes. They were most abundant in the dark chestnut and carbonate soil. Their numbers increased with the depth of soil, whereas the number of species diminished or remained unchanged. Halophylic and facultative halophylic actinomycetes were isolated from salt meadows; the former develop better with 1.5 to 3 per cent NaCl or Na_2SO_4 in the medium, and the latter without additional salt.

The effect of partial sterilization of soil upon the changes in its actinomycete population was studied by Waksman and Starkey (1923). They found that this treatment of the soil brought about a considerable change in its microbiological population, the actinomycetes behaving differently from the fungi and bacteria, depending upon the nature of the treatment and the organic matter content of the soil. In general, the actinomycetes were among the more persistent forms.

In a study of the survival and growth of *Nocardia* in the soil, Brown (1958) found that in partially sterilized soil wide fluctuations occurred during the first month, followed by a steadying in count, and at the end of the year *N. cellulans* was still present in high numbers. In the untreated soil *N. cellulans* disappeared in 6 months. A regular cyclical morphological development of the organism was observed, peak counts corresponding to the rod form and troughs to the mycelial form, and the steadying in count to the rod form.

Numerous attempts have been made to classify streptomycetes by their growth characteristics on agar media in which a soil has been plated out. Misiek (1949) found that variability and inconsistency in diffusible pigment production and carbon utilization rendered their use for identification purposes unfeasible. Of the various properties considered, pigmentation patterns of vegetative mycelium, and the appearance of aerial hyphae and spores were less variable, and thus more conducive to a study of this nature. An examination of 1,510 isolates for their carbon utilization gave positive results that varied from 5 per cent for sorbitol to 98 per cent for glycerol, with inulin, sucrose, arabinose, rhamnose, raffinose, xylose, mannitol, lactose, cellobiose, levulose, starch, dextrin, galactose, maltose, and glucose in increasing order.

The streptomyces isolated from soil at depths of 8 and 16 inches were the same as those found near the surface, and were probably transported downward by the percolation of surface water. The presence of large numbers of a particular variety in a soil gave a definite indication of its predominance. A significant correlation was believed to exist between different soil samples within a soil series and between similar series, and some types of predominating streptomyces populations inhabiting these soils. No correlation could be made between topsoil reaction, soil series, and predominating varieties of streptomyces cultures. Certain predominating varieties in a given soil were found to appear, disappear, and reappear during certain periods of the year, a phenomenon more apparent in some soils than in others.

TABLE 6

Distribution of actinomycetes in two forest soils during different seasons of year (Cobb)

Counts of organisms represent numbers per gram dry soil

Date (1930–1931)	Hemlock topsoil	Hemlock subsoil	Deciduous topsoil	Deciduous subsoil
January*	400,000	196,000	188,000	251,745
February*	412,500	150,370	761,000	248,120
March	105,880	140,800	472,400	172,400
April	215,050	122,440	555,550	166,665
May	164,940	0	295,420	292,515
June	188,675	90,300	233,575	209,150
October	8,845	14,860	76,470	51,140
November	55,425	14,705	33,330	70,175
December	61,855	39,215	73,530	153,330
January*	206,180	91,500	49,250	116,125
February*	75.000	42,380	213,675	84,510
March	28,450	148,645	620,435	13,245

* Soil frozen.

Numerous other studies were made of the total abundance and occurrence of specific types of actinomycetes, especially streptomyces, in different parts of the world. They were reported in great abundance from soils as far apart as the United States (Starkey, Vandecaveye *et al.*, Cobb), China (Eggleton), Formosa (Adachi), Japan, South America, and India. Their great abundance in peat soils (Zimenka) must be clearly differentiated from their occurrence in peat bogs. They are largely limited to the very top layer of lowmoor peat bogs, and are absent entirely from natural highmoor peats; in the latter, both the combined acidity and moisture saturation are the factors responsible for their absence (Waksman and Purvis).

Mycelial versus spore stages. The question of whether actinomycetes occur in the soil in the mycelial or the spore stage has been given considerable attention. Conn was the first to demonstrate, by the direct staining method, that actinomycetes occur in the soil as vegetative mycelium. Subrahmanyan, on the other hand, found that actinomycetes occur in the soil largely as spores; vegetative mycelium was said to be present on undecomposed plant residues but not in the soil itself.

Jensen, in 1943, made a comparative examination of the relative abundance of actinomycetes in the form of vegetative growth and as spores, using the microscopic and plate methods. He found that vegetative mycelium developed most abundantly at 28 to 37°C; at 15°C growth was slower but eventually reached the same density as at the higher temperatures. Vegetative growth declined more or less rapidly, especially at the higher temperatures, whereas the plate counts remained at a high level for some time after reaching a maximum; this was said to be due to the increasing fragmentation of the hyphae and progressive formation of spores.

As pointed out previously (Chapter 2), Skinner, using the shaken soil suspension technique, demonstrated the presence of mycelial filaments in the soil. On continued shaking, the number of colonies obtained from a given quantity of soil was increased to a certain maximum; then the number diminished as a result of destruction of the cells.

Lutman, Livingston, and Schmidt concluded that actinomycetes are of great importance in the soil, especially in respect to changes they bring about in the transformation of the soil organic matter. They appeared to exist in the soil in the form of mycelial fragments, probably attached to decomposing organic particles, although in soil smears they were found in a free state. The numbers of colonies obtained on soil plates (Table 7) corresponded closely to the pieces of mycelium that were seen on smears by the direct count method. It was concluded that the numbers of colonies of actinomycetes that develop on an agar plate from fresh soil dilutions represent the total numbers of actinomycetes present mostly as bits of vegetative hyphae; when the soil dilutions were heated to a point at which the mycelium was destroyed and only the spores survived, the numbers of colonies obtained were only a fraction of a per cent.

Jensen (1934–1936) found that the development of actinomycetes in soils treated with various organic materials was favored by a high temperature and by a low moisture content. Decreasing soil moisture and increasing soil temperature stimulated the growth of actinomycetes over that of bacteria. Although neutral or alkaline reactions are definitely favorable to the growth of actinomycetes, Jensen found these organisms in fairly acid soils (pH 3.4–4.1).

Cholodny reported that a low moisture content favored vegetative growth of actinomycetes. von Plotho also observed that a dry atmosphere stimulated spore production by actinomycetes. According to Porchet, treatment of forest soil with formalin (1 per cent) does not destroy the actinomycete population. Warren *et al.* found that soils in which the plants were sprayed with 2,4-D allowed extensive growth of actinomycetes that possessed strong antifungal properties. The effect of enrichment of soil with bacteria leads to extensive actinomycete development; such organisms are not necessarily endowed

TABLE 7

Monthly counts of bacteria and actinomycetes in a greenhouse soil (Lutman, Livingston, and Schmidt)

	Soil moisture, per cent	Total organisms, thousands per gm of dry soil	Actinomycetes, thousands per gm	Actinomycetes, per cent of total organisms
January	26.5	21,450	2,832	13.2
February	36.7	26,629	5,308	19.9
March	22.0	15,554	3,917	25.2
April	20.3	14,167	3,074	21.7
May	16.8	12,124	2,094	17.3
June	16.1	12,289	2,157	17.5
July	14.4	10,612	2,072	19.5
August	15.0	11,407	1,808	15.9
September	13.9	8,839	1,940	21.9
October	19.0	12,282	2,640	21.5
November	20.0	17,764	3,433	18.7
December	24.4	13,261	2,660	19.3

with special antibiotic-producing properties. Their excessive growth is no doubt due to the introduction of a fresh supply of available nutrients in the form of bacterial cells.

An examination of soils enriched with streptomyces spores, using the Rossi-Cholodny contact slide method, revealed that those spores remained in the soil largely in an ungerminated state. They grew only on the rim of the soil adhering to the glass and in the dead bodies of soil amoebae (Pfennig).

Nature of the soil actinomycete population. No attempt will be made to review here in detail the numerous other investigations on the abundance of actinomycetes in the soil, as determined by the plate method. Suffice to say that all these studies established the fact that actinomycetes form an essential constituent part of the soil microbiological population. Although some assumptions have been made that colony counts of bacteria, actinomycetes, and fungi represent the numbers of spores in the soil and not of active organisms, the evidence presented here proves the contrary. By separating the nocardias (designated as proactinomycetes) from the streptomyces (designated as actinomycetes), Topping failed completely to recognize the nature of these organisms, namely, their vegetative *versus* sporulating growth, and the possible confusion between them; he did recognize, however, the nocardias and nocardia-like organisms as members of the native or "autochthonous" microflora of the soil. Further information on the occurrence of nocardias in the soil is found in the work of Frey and Hagan (1931) and Gordon and Hagan (1937).

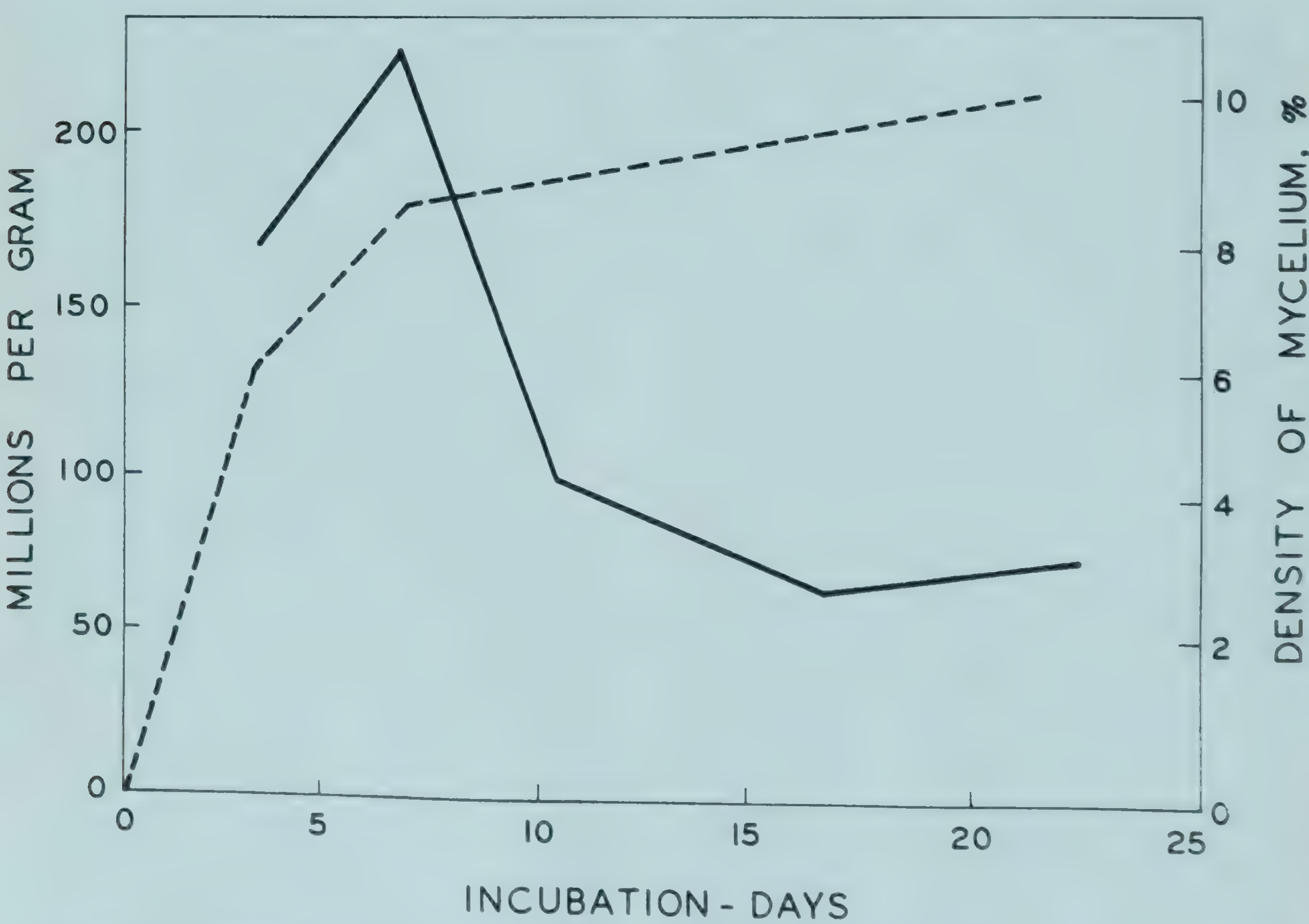


FIGURE 19. Comparison between density of vegetative mycelium and plate counts of actinomycetes in soil. Continuous line: density of mycelium; broken line: plate counts (Reproduced from: Jensen, H. L., Proc. Linnean Soc. N. S. Wales 68: 69, 1943).

The addition of organic matter to the soil has a marked stimulating effect upon the development of actinomycetes, as illustrated by Waksman and Starkey (1924).

One more problem pertaining to the survival of actinomycetes in the soil must be considered, namely, the survival of organisms added to the soil. Fousek reported that addition of cultures of actinomycetes to soils rich in organic matter hastened decomposition of the humus and increased liberation of the nutrients. It is a well established fact that when lowmoor peat soils are freshly drained and cultivated, actinomycetes will develop at a rapid rate in such soils (Waksman and Purvis, 1932). If the potato scab organisms are able to make an entrance into peat-rich soils, and potatoes are planted in such soils, the resulting scabby potatoes may play havoc with the crop. If cultures of actinomycetes are added to ordinary soils, however, they tend to die out rapidly, as demonstrated by Waksman and Woodruff (1940). This has been explained by the complex antagonistic interrelations among the various groups of microorganisms inhabiting the soil.

Among the most important factors controlling the abundance of actinomycetes in the soil, one must recognize, (a) the nature and abundance of the organic matter, (b) the reaction, (c) the relative moisture content, (d) the temperature, (e) the aeration of the soil or the oxygen supply, and, finally, (f) the soil vegetation (Rouatt *et al.*, 1951).

In general, actinomycetes are less favored by a higher moisture content than are the bacteria. They are able to grow well at a relatively low moisture, even at 15 to 20 per cent of the moisture-holding capacity of the soil. Most of the bacteria, which grow best at 50 to 65 per cent moisture-holding capacity, do not develop at all under those conditions.

Although actinomycetes are as a rule cosmopolitan in their distribution, since they are found universally, the specific substrate and environment greatly influence their nature. This is true, for example, of soils in which successive crops of potatoes have been grown.

Further studies on the wide distribution of actinomycetes in soil have been made by Négre (Sahara soils), Jensen (1930, 1931, 1934, 1936), Waksman (1932), Adachi and Imamura (1933), Hopf, Strutz (1952), Jagnow (1957), and others. Detailed studies of the occurrence in soil of actinomycetes possessing antagonistic properties against other microorganisms and capable of producing antibiotic substances are presented in Chapters 14 and 15.

Occurrence of Actinomycetes in the Sea

Only very few reports are available concerning the occurrence of actinomycetes in sea water and sea bottoms. Their occasional presence in this environment was usually believed to be due to soil contamination, or to their presence on algal material floating on the surface of the sea, or to the fact that the samples of water were obtained near the docks (Zobell and Upham). Sea water enriched with petroleum hydrocarbons permitted the development of nocardias and micromonosporas (Zobell *et al.*).

Humm and Shepard reported (1946) the isolation of several agar-decomposing actinomycetes from marine material. Some appeared to belong to the genus *Nocardia* and others to *Streptomyces*. Freitas and Bat (1954) also isolated members of these two genera from deteriorating fish nets and cordage.

Schwartz and Siebert and Schwartz (1956) made a detailed study of the occurrence of actinomycetes in marine sediments. Because of the resistance of these organisms to high salt concentrations, one would expect to find them in such sediments. When filter paper was placed in contact with marine sediments the development of various species of actinomycetes was observed, including *S. albus*, *S. bobiliae*, *S. rubescens*, and *N. cuniculi*. Grein and Myers also demonstrated the occurrence of various actinomycetes in marine sediments. They suggested that this is due to their salt tolerance and to their ability to survive for considerable periods of time under marine conditions.

Various observations have been made concerning the ability of certain types of actinomycetes to become adapted to high salt environments. This adaptability is particularly marked in organisms found in salt lake muds. Nadson observed, in 1903, the presence of actinomycetes in curative salt muds; he believed that they were concerned with the decomposition of proteins, liberation of ammonia and hydrogen sulfide, and resulting in the precipitation of $CaCO_3$. Sawjalow claimed to have found an actinomycete, designated as *A. pelogenes*, in black mud, frequently used for curative purposes, in the region of Odessa; the black color of the mud was said to be due to the biological reduction of the sulfate by the actinomycete. It is now believed, however, that this was a bacterial culture. Issatchenko (1927) suggested that the low salt concentration of the lakes studied by Nadson may have played an important function in favoring the activities of the actinomycetes. Issatchenko himself found these organisms also in lakes with high salt concentrations. However, they grew in culture only with a much lower concentration of salt, namely 10 per cent NaCl. Under these conditions, proteins were actively decomposed with the formation of ammonia and H_2S. Issatchenko explained the frequent occurrence of actinomycetes in lake muds with high salt concentration as due to the survival of contaminants from surrounding fields. Further studies on the occurrence of actinomycetes in salt lakes were made by Issatchenko (1938) and Rubentchick (1948).

Occurrence of Actinomycetes in Lake and River Waters

Fresh water lakes contain an abundance of actinomycetes. Kedzior first established in 1896 that thermophilic actinomycetes are found in river water. They were also found in sewage. They grew well at 60°C.

Price-Jones described in 1900 three cultures of actinomycetes that would now be classified in the genus *Streptomyces*, which he isolated from lake and river water.

The numbers of actinomycetes in fresh waters are not very large, however, as shown by Potter and Baker (1956). Most of the species isolated appear to be largely members of the genus *Micromonospora*. These organisms produce on the plates hard colonies pigmented orange to pink, and lacking the typical aerial mycelium of the *Streptomyces*.

The *Micromonospora* group was also found in great abundance in the bottom deposits of Wisconsin lakes, especially in profundal zones rich in organic matter. Their abundance as related to the bacteria found in the same samples varied from 2.9 to 48.5 per cent with an average of 13.4 per cent. The southern lakes showed larger numbers than the northern lakes. The lake waters had only negligible numbers of micromonosporas unless the bottom deposits had been agitated and the organisms distributed through the overlying water layers. Vertical distribution through the bottom cores showed a decrease in numbers as one proceeded downward. The soils adjacent to the lakes had a few micromonosporas, but large numbers of streptomyces (Colmer and McCoy, 1943).

Umbreit and McCoy reported that 10 to 20 per cent of the total microbial population of the water comprise micromonosporas. At times they made up 40 to 50 per cent of the total numbers of colonies developing on the plate. The same was found to be true of the lake mud bottoms. In some cases as many as 100,000 cells of micromonospora were found per milliliter of lake mud, amounting to 45 per cent of the total microbial population. With an increase in the depth of the bottom material there was an increase in the numbers and percentages (up to 60 to 70 per cent) of actinomycetes. The conclusion was reached that the genus *Micromonospora* represents a truly indigenous group of microbial inhabitants of waters and bottom deposits of inland lakes. Since these organisms are aerobic and grow very slowly, it was at first believed that their role in the decomposition of organic matter in water basins was only a minor one. Their ability to attack resistant organic materials, such as lignins, suggested, however, a potentially important function for these organisms.

Erikson isolated 10 strains of *Micromonospora* from lake mud and lake water. These were capable of growing on a large variety of resistant organic compounds, such as chitin, cellulose, and, to a lesser degree, lignin. It was suggested that these organisms play a significant part in lacustrine ecology, being adapted to life under aquatic conditions and being able to utilize resistant substances of the type found in lake mud.

Actinomycetes are also found in river waters and in river bottoms. As a consequence, a serious problem may arise, namely, the imparting to the water of the odor characteristic of cultures of actinomycetes. This odor renders the water unsuitable for drinking purposes. Adams drew attention to the fact that the "earthy" taste of the water of the river Nile was due to actinomycetes that inhabit or "contaminate" the water. Burger and Thomas suggested that the peculiar taste of the water was due to the decomposition of plants growing in the waters, the actinomycetes forming merely a small part of the total microbiological population.

The role of the actinomycetes in the production of odoriferous substances in the water has received considerable attention. Putilina examined the abundance of these organisms in the waters and sediments of the Don basin. Their numbers in the bottom material were 10 to 1000 times greater than those in the water. They were believed to be responsible for the unpalatable odors imparted to the Don waters. When the organisms were isolated in pure culture, similar odors were produced in artificial media. Egorova and Issatchenko also found an extensive population of actinomycetes in river bottom deposits. The numbers varied from 36,585 to more than a million per gram of dry material. Large numbers were recorded even at a depth of 20 cm. Only few organisms were found in the water itself. When some of the water and a layer of bottom material were placed in cylinders, sterilized, and inoculated with pure cultures of actinomycetes, excellent growth was obtained in a short time. The earthy smell and the unpleasant flavor of the Moscow river water was ascribed to the multiplication of actinomycetes in the bottom muds, especially during the summer and fall months. They were largely of the streptomyces type. The sandy soils along the river banks contained a larger number of actinomycetes. The odor produced by them is washed out by rains and carried into the river. This takes place especially after the soil undergoes a spell of dry or freezing weather. The numbers of actinomycetes increase particularly in autumn. The passage of the odoriferous substance into the water depends also on the nature and adsorbing capacity of the bottom material: sandy bottoms give a marked odor, and clay bottoms only little odor because of its adsorption on the clay (Issatchenko). Thaysen has also made a study of the relation of actinomycetes to the odor of river water, as shown later in this chapter. Numerous other studies have been made on the occurrence of actinomycetes in different kinds of waters, to which they can impart odors and tastes

Table 8

Microbiological population of an undrained peat bog in Forida (Waksman and Purvis)

Numbers in 1 gm of moist* peat, in thousands

Depth of peat, cm	Aerobic bacteria	Actino-mycetes	Fungi	Anaerobic bacteria
2–20	890	370	20	120
23	960	290	10	180
45	410	100	7	180
75	18	13	0.3	16
120	30	0.3	0	75
165	235	3.3	0	380

* Moisture content varied from 80.1 to 87.4 per cent.

(Silvey and Roach). They also occur in chlorinated waters (Adams, 1929).

Waksman and Purvis (1932) made a study of the microbiological population of an undrained peat bog in Florida. The numbers of actinomycetes were found (Table 8) to decrease rapidly with depth.

Hvid-Hansen (1951) reported the presence of an anaerobic *Actinomyces* in ground water containing hydrogen sulfide. This organism produced in an organic medium formic acid, propionic acid, and lactic acid. Since sulfate-reducing bacteria are able to use these compounds as hydrogen donors in an otherwise autotrophic medium, a close symbiosis was assumed to exist between the anaerobic actinomyces and the sulfate-reducing bacteria. Six strains of the organism were isolated and described. Further information on this organism, named *A. hanseni*, is given in Chapter 24, Vol. II.

The occurrence in fresh water of organisms belonging to the genera *Actinoplanes* and *Streptosporangium* has been reported by Couch.

Occurrence of Actinomycetes in Stable Manures and Composts

Just as the actinomycete population of the soil is characterized chiefly by the genus *Streptomyces*, as lake waters and lake bottoms are characterized by a population of *Micromonospora*, and as the actinomycetes causing human and animal diseases are limited primarily to the genera *Actinomyces* and *Nocardia*,—so the actinomycetes living in composts of stable manures and plant residues, especially high temperature composts, are limited almost entirely to certain specific genera related to *Streptomyces* and *Micromonospora*. Composts frequently attain temperatures of 50 to 65°C and may even reach 80°C. This is due to the evolution of heat resulting from the activities of the microorganisms bringing about the decomposition of the plant and animal residues, especially the carbohydrates and the proteins. The compost must be thoroughly aerated to bring this about, since an anaerobic environment does not favor rapid decomposition.

The occurrence of actinomycetes in large quantities in stable manures has long been recognized. It is sufficient to mention the early work of Miquel in 1879, of Tsiklinsky in 1899, and of Price-Jones in 1900. Tsiklinsky isolated two thermophilic actinomycetes from manures; one was a typical streptomyces and the other a micromonospora-like organism, which was designated *Thermoactinomyces vulgaris*, since it grew at 48 to 68°C, with an optimum at 57°C. Miehe, in his work on the "self-heating of hay," suggested that actinomycetes are, in general, characteristic of decomposing plant residues at high temperatures; hot composts were believed to be the natural substrates for thermophilic actinomycetes. Schütze, Lieske, and others also demonstrated the presence of actinomycetes in decomposing and heating hay and in feces.

Waksman, Umbreit, and Cordon found an extensive population of facultative thermophilic actinomycetes in various soils, especially those treated with stable manures. Composts of horse manure kept at 50 and 65°C developed an extensive and characteristic population of these organisms. Six distinct types were recognized, belonging to the genera *Thermoactinomyces* and *Micromonospora.*

That the intestinal canal is the source of thermophilic actinomycetes was demonstrated by Tsiklinsky and Bruini. The occurrence of thermophilic forms in soil has generally been correlated with the application of stable manures (Mishustin). Henssen recently recorded the isolation from manures and composts of 11 thermophilic species of actinomycetes placed in five genera, as shown in detail in Chapter 28, Vol. II.

Sewage is frequently found to contain actinomycetes. As pointed out previously, Kedzior was the first to demonstrate the presence of thermophilic actinomycetes in sewage. Brussoff isolated an organism, designated as *A. cloacae,* definitely a streptomyces, from the slime of the Aachen purification system. This organism was found capable of decomposing cellulose. Brussoff accepted Beijerinck's and Krainsky's concepts of the omnivorous nature of the actinomycetes. Still he believed that his organism was capable of fixing nitrogen, a fact not usually accepted at present, although it grew better on nitrogen-containing media.

Occurrence of Actinomycetes in the Atmosphere

Actinomycetes occur abundantly in the atmosphere, both in the form of mycelium and as spores. This can easily be illustrated by exposing agar or gelatin plates to the air for a few minutes or by collecting some of the dust and analyzing it. Actinomycetes are also universally found on rocks, plants, animals, clothing, food, and other surfaces exposed to the atmosphere. Because of the ability of actinomycetes to withstand desiccation, their presence on such surfaces results from the breaking up of fine soil particles suspended in the atmosphere and the drying of water drops. Wind currents are also largely responsible for their wide distribution.

The presence of microscopic particles of dust in the air was known to the ancients. The Roman poet Lucretius first observed such particles by passing a ray of sunlight through a darkened room. With the birth of modern microbiology, ever-growing attention was paid to the microbes found in the dust. Leeuwenhoek observed them in rain drops; Ehrenburg found them in rain water and in snow; J. Tyndall saw them directly in the dust. The whole question of spontaneous generation, which aroused so much attention and which was settled largely through the work of Pasteur, had a great deal to do with the interest in the microbial population of the atmosphere. The question of contagion and the problems of epidemics appeared to be at that time closely related to the air as a carrier of microbial agents. More recently, the bearing of the dust microflora upon the problem of allergy has begun to receive considerable attention.

Foulerton and Price-Jones spoke of the isolation of actinomycetes (*Streptothrix erythrea*) as air contaminants. They also mentioned the isolation of actinomycete cultures (*St. leucea* and *St. leucea saprophytica*) from sewage and from drinking water.

Lidwell suggested that the problem of sampling air for microbes comprises collection of the sample from the atmosphere and the determination of the microbial cells in the sample, their enumeration, and their classification. Methods of air sampling include: (a) sedimentation, usually into open dishes; (b) filtration, through wool, sand, or other materials; (c) centrifugation; (d) collection in liquid by bubbling air through it; (e) electrostatic precipitation.

Special apparatus was constructed for collecting atmospheric dust. Exposures were made at different altitudes and under different conditions. The first comprehensive study was undertaken by Miquel in 1883.

Among the many organisms that he found in the atmosphere, some, frequently designated as branching bacilli and cladothrix, no doubt belonged to the actinomycetes.

Rossi-Doria, in 1891, made a detailed investigation of the actinomycetes in the air. He was the first to use the specific name *alba* for actinomycete species. Since the genus to which this species belongs is now known as *Streptomyces*, the *Streptothrix alba* of Rossi-Doria is now recognized as the type species *Streptomyces* or *S. albus*. Among the other species listed by him as occurring widely in the atmosphere, were *S. violacea*, *S. albidoflava*, *S. nigra*, *S. carnea*, *S. aurantiaca*, and *S. chromogena*.

In 1903, Beijerinck and van Delden isolated a culture of an organism which was believed capable of purifying laboratory air rich in carbon monoxide. They suggested that the culture used CO gas as a source of energy, the CO being oxidized thereby to CO_2. The organism was described as a *Bacillus*, under the name of *B. oligocarbophilus*. Lantzsch repeated these studies later and found that the organism was actually a true actinomycete; it could assimilate not only CO_2 but also higher aliphatic hydrocarbons, except benzol and xylol. He, therefore, changed its name to *A. oligocarbophilus*.

Numerous other investigators observed the occurrence of actinomycetes in the air. Caminiti found the organisms in hospital air, Barthel observed them in the dust of stables, Bellisari reported their occurrence on the dust covering cereals.

Occurrence of Actinomycetes on Food Products

Actinomycetes are found extensively on and in various food products. In some cases they are able to produce extensive growth and have, therefore, been recognized as the cause of considerable spoilage. The damage was believed to be due not so much to the actual destruction of the foodstuffs as to the undesirable musty odors the organisms impart to the food. The characteristic odors produced by actinomycetes have attracted a great deal of attention. Rullmann first believed that the odor was characteristic only of a certain species that he designated as *A. odorifer*. The odor itself was referred to frequently as "earthy," since it is similar to that of well-aerated soil.

Actinomycetes usually develop upon foodstuffs under conditions not very favorable to either fungi or bacteria, corresponding to fairly high temperatures and low moisture contents. At a moisture suboptimum for development of other spoilage-producing microorganisms and at too high temperatures, food materials may be subject to attack by actinomycetes, especially under aerated conditions.

Actinomycetes are responsible for undesirable odors and flavors produced in milk. Barthel was primarily concerned with the occurrence of microorganisms in fresh milk and in the cow's udder. He isolated two cultures designated as *A. albus* and *A. chromogenes*, both typical *Streptomyces* species, and came to the conclusion that these come from the air. Fellers directed attention to the possible damage to milk due to the undesirable odors and flavors. Hlavackova (1951) reported that as many as 16.3 per cent of samples of raw milk may contain actinomycetes. Their presence was considered as an indicator of the degree of contamination of the milk with dust and excreta. These organisms may be pigmented and may thus cause additional damage to milk and butter. If the milk is insufficiently or improperly pasteurized, the actinomycetes will survive. Gratz and Vas isolated two streptomyces cultures from Littauer cheese. Jensen recorded their occurrence in butter. Chatterjee found an actinomyces in fermented milk in India.

The earthy or "muddy taint" occasionally found in fish was studied in detail by Thaysen. Salmon caught in some of the richest salmon rivers in Great Britain were found contaminated with this odor, which became so noticeable when the fish were boiled that they were inedible. The odoriferous substance was soluble in water and volatile in steam. The river water itself where the particular fish were caught also had this "earthy" odor. It was strong along the banks where mud overgrown with reeds had accumulated. Thaysen plated out the submerged river mud; he suggested that the actinomycete colonies thus obtained were not derived from spores but represented active "foci" of growth, as shown in Table 9. On comparing similar numbers from submerged material in a river that did not have the "earthy" odor, Thaysen found 13,000 actinomycete colonies per gram of submerged mud, 52,000 per gram of waterlogged vegetation, and 244,000 per gram of vegetation removed from the dry bank of the river. None of these cultures was of the odoriferous type. In the contaminated river, the actinomycetes represented "an abnormally high proportion in the microflora."

TABLE 9

Number of actinomycete "foci" in submerged river bank mud (Thaysen)

Left bank of river, odor strong					Right bank of river, odor slight			
Yards below tidal limit								
120	200	220	600	1100	120	220	600	1100
Thousands of actinomycetes per gm of material								
280	220	609	330	400	8	0:1(?)	500	362

Among the other food products subject to considerable damage from the occurrence of actinomycetes is the cacao bean. In 1927 Ciferri undertook a study of the causative agents of the musty odor of these beans in the Dominican Republic. He found this odor to be associated with the occurrence of actinomycetes; the commonest form was a variety of *S. albus*. Bunting, in 1932, isolated three cultures of actinomycetes, described under a species name *S. cacaoi*. The pungent musty odor of the cacao was found to be caused by these organisms.

According to Haines (1932), actinomycetes occur commonly in commercial cold stores. They were isolated from the walls and especially from the straw on the floor of the stores.

Among the other food products that may be contaminated with actinomycetes, it is sufficient to mention rum, as first pointed out by Price-Jones.

Occurrence of Actinomycetes on and in Plants

Actinomycetes occur universally on the surface of plants and sometimes even in various parts of the plants themselves. This is true, for example, of potatoes. Some of these organisms are saprophytic in nature and others are pathogenic, being responsible for the causation of specific plant diseases, as pointed out in Chapter 18.

Various theories have been proposed concerning the function of the actinomycetes in the plants, since they are known to occur in the outer layers of roots and tubers. Beijerinck found various plants to be superficially infected with actinomycetes. In 1903, Petri isolated an actinomycete culture from the roots of strawberry plants; although this organism could be inoculated into fresh plants, it was considered to be a saprophyte, since the plants were in a healthy state even after 6 months. Lutman observed actinomycete filaments growing along the cell walls of potatoes and other plants; the filaments were branching and were passing into the cell lumen, twisting and bending in tortuous paths; the stems above ground, the leaves, and the flowers were also completely infected. Lutman postulated the theory that the cells of the actinomycetes take part in the synthesis of alkaloids and proteins in the plant, as well as in the dissolution of the pectins. Their role in tuber formation and in plant growth in general was suggested.

The isolation of various organisms from diseased plant tubers and other plant tissues has been studied in detail by Peklo, Millard and Burr, and numerous others concerned with the causation of plant diseases by actinomycetes. The earlier work of Arzberger and Peklo on "plant actinomycoses," and the possible role of actinomycetes in the tuberization of species of *Myrica*, appeared also to have a bearing upon this problem.

Occurrence of Actinomycetes on and in Animal Bodies

The etiology of actinomycotic infections in man and in animals is closely bound with the occurrence of specific organisms at the sight of infection. Whether actinomycosis is a result of exogenous infection due to the consumption, by animals, of grasses and foodstuffs containing actinomyces spores and mycelium, or of endogenous infection due to the presence of these spores and mycelium as regular inhabitants of the healthy mouth aroused, in the past, a great deal of discussion (Lentze). The history of the causative agent of actinomycosis is closely bound with the occurrence of actinomycetes at the sight of infection, since considerable difficulty has been experienced in the isolation of the causative agent of the disease and in bringing about experimental infections by these organisms.

Bollinger demonstrated that the common types of bovine actinomycosis are due to an organism named by Harz in 1877 *Actinomyces bovis*. Wolff and Israel are credited with the first isolation from maxillary actinomycosis of cattle of a microaerophilic strain of *A. bovis*, which is now considered as the true etiologic agent of the disease. Apparently, actinomycosis is an ancient disease, since evidences of it have been found in fossilized animals (Moodie).

According to Rosebury and Sonnenwirth, the only actinomycetes indigenous to man are the anaerobic forms which are grouped in a single species *A. israeli*. They are found in the mouth, pharynx, intestine, and actinomycotic lesions. They are considered as strict parasites of man and many animals. The separation between the smooth *A. bovis* found in cattle and rough *A. israeli* in man may not be tenable.

The further assumption that the etiologic agent *A. bovis* propagates in the soil and that cattle become infected while grazing upon grass has been another source of error. Emmons pointed out that the true etiologic agent *A. bovis* has not yet been isolated from a natural habitat outside the animal body. It has been assumed that this organism has both a saprophytic and parasitic existence within the body itself. Emmons obtained pure cultures of the microaerophilic *Actinomyces* from the surface of discolored teeth, from carious teeth, and from tonsilar crypts. When first isolated, these cultures grew more rapidly and the hyphae were coarser than those of the strains isolated from clinical actinomycosis, but upon repeated subculture, they became similar to *A. bovis* in morphology. Lord demonstrated the presence of actinomycetes in the contents of carious teeth and in the crypts of tonsils; he considered the buccal cavity as the source of infection. Further studies of the occurrence of actinomycetes in the oral cavity have been made by Naeslund and by Ludwig and Sullivan.

Actinomycetes have also been found extensively in numerous other organs and excreta of the human and animal body, such as the fresh excreta of suckling animals (Moro) and the intestinal canal of healthy animals (Hopffe). Fischer (1915) found actinomycetes (*S. albus*) to occur abundantly in the caecum, in the large intestine, in the rumen, honeycomb, and other stomachs of ruminants, and in the rectum; he suggested that these organisms are obligate inhabitants of the intestinal system of animals. Further studies on the actinomycetes of the intestinal population of horses are found in the work of Hoffe, and of man in the work of Breit.

Various actinomycetes are frequently found in organs of certain insects. An organism designated as *Nocardia rhodnii*, isolated from the reduvid bug, *Rhodnius prolixus* (Erikson, 1935), is said to be taken up by the young nymph from the contaminated surface of the egg or to be transmitted to the insect by the dry excreta of the insects. When the insect is freed of the actinomycete by sterilizing the surface of the egg and by feeding the adult with suitable precautions, sterile insects are produced. These grow and moult normally only for a certain period, but they are usually incapable of reproduction. When the insects are reinoculated with the actinomycete cultures, normal growth, moulting, and egg production will result.

The occurrence and isolation of actinomycetes from animal diseases have already been mentioned (Chapter 1) and will be discussed in greater detail later (Chapter 17). Many of the organisms reported to have been isolated from disease conditions, beginning with those of Bostroem and ending with those of Erikson (1935), may not be the causative agents of the disease, since only few animal experiments were carried out, in most cases, to confirm this assumption.

Actinomycetes and Geologic Formations

The role played by actinomycetes in geologic formations is not yet sufficiently clear, although some evidence has been submitted on the question, as will be shown later (Chapter 9).

Interrelationships among Microorganisms in Natural Substrates

The remarkable progress made in recent years on the production of antibiotics by soil microorganisms, especially actinomycetes, has aroused considerable interest in the possible effect of such antibiotics on the microbiological population of the soil. Some investigators, notably Krassilnikov, were inclined to see in these antibiotics mechanisms that the soil microorganisms use in competition for existence. Waksman considered these concepts as purely speculative in nature. In support of this conclusion, the following evidence was submitted.

1. Actinomycetes occur in the complex natural substrate in a mixed microbiological population. This population consists of numerous groups of microbes, which range from the ultramicroscopic forms, through the bacteria, actinomycetes, and filamentous fungi, to the complex mycelium of the higher or mushroom fungi, the protozoa, and various small invertebrates. Each of these groups is represented in nature, especially in the soil and in water basins, by many species and varieties, which have the capacity to bring about many complex chemical reactions. The ability to produce antibiotics is only one such property and is limited to certain kinds of microbes, when these are grown in a pure state, in an artificial environment, and in a special medium.

2. For the formation of antibiotics in significant concentrations in such an artificial medium, certain nutrients, characteristic for each organism and comprising largely proteins or amino acids, sugars and other compounds, must be present. Such nutrients are seldom found in the soil in sufficient concentrations to enable even the antibiotic-producing organisms to dominate the environment or to produce measurable quantities of the antibiotics.

3. All the experimental evidence so far

obtained tends to establish the fact that in a natural soil, in the presence of the numerous competing forms of life, no antibiotic is produced; were the reverse to be the case, the antibiotics would have played an important part in the distribution of various groups of microorganisms in the soil. Only among the actinomycetes do we find a large proportion of organisms capable of producing antibiotics. One could have expected a much greater number of organisms in the soil, notably among the bacteria, to be resistant to the particular antibiotics.

4. All attempts to isolate specific antibiotics from, or to demonstrate their presence in, the soil have so far failed. In those few instances where antimicrobial activities have been demonstrated in the soil, it is still uncertain whether antibiotics are present there as such, and if so, whether they are of microbial or plant origin, how they have been formed there, and of what significance they are in modifying the native population of the soil. Such activities may actually be due to various plant products left in the soil as a result of the decomposition of plant residues, such as tannins, oils, resins, or lignins and lignin derivatives.

5. Enrichment of a fresh natural soil with living cultures of microorganisms has failed to stimulate or favor the selective development of other organisms that would be capable of producing antibiotics active against the organisms introduced. Antibiotics are not enzymatic systems that are stimulated by the addition of special nutrients. Earlier claims of the favorable effects of additions of microbial cultures in enriching the development of antibiotic-producing organisms have not been confirmed on further study.

6. When pathogenic and even saprophytic bacteria are introduced into fresh soil or into fresh sea water, they do not multiply in the new environment, but tend to die out rapidly. Although this was originally ascribed to the presence in the soil or in the sea of antibacterial substances, comparable to antibiotics, the actual facts have not substantiated this explanation. Such substances are usually removed on sterilization, whereas many of the antibiotics are resistant to heat. It may, of course, be argued that the disappearance of cultures of organisms added to soil or water is due not so much to the presence of antibacterial substances as to the competition of the organisms capable of producing such substances. This explanation begs the main question, however, whether the formation and accumulation of antibiotics can take place at all under natural conditions.

The following illustration may be presented in support of the foregoing statements. Considerable information has recently been accumulated on the disturbed microbiological population in the animal digestive system due to the consumption of antibiotics. Of particular interest in this connection are the effects of products of metabolism of actinomycetes, such as streptomycin, chloramphenicol, and the tetracyclines, upon the intestinal population of animals. Streptomycin is known to favor the rapid development of resistance among the sensitive bacteria. Baumgärtel succeeded in extracting desoxyribonucleic acid from resistant strains and transplanting it to sensitive strains. Sensitivity and resistance of an organism to a given antibiotic thus depend upon the chemical composition and the metabolic mechanisms of the organisms concerned rather than upon a dialectic concept of "struggle for existence." The possibility that the localized production of antibiotics on plant residues may be of ecological significance is not excluded, however, as shown by Brian (1957).

CHAPTER 4

Nomenclature and General Systems of Classification

Problems of Generic Nomenclature

The first organism belonging to the actinomycetes that was ever recorded and to which both a generic and a specific name were given was described by Cohn in 1875 as *Streptothrix Foersteri*. Unfortunately, this organism was not isolated in pure culture, nor was it described sufficiently for identification. Further, the generic name given to it by Cohn had been preempted for a true fungus (Corda, 1839), so that it could no longer be considered as valid. There was not even general agreement, subsequently, whether the organism studied by Cohn was pathogenic or nonpathogenic. In spite of all these objections, the name Streptothrix continued to be used for many years, until it was finally discarded on the basis of lack of validity, in accordance with the rules of botanical nomenclature.

The second actinomycete was studied and described by Harz in 1877 as *Actinomyces bovis*. No one now questions the fact that this organism was associated with a disease condition known as "lumpy jaw" of cattle, since designated as bovine actinomycosis. Unfortunately, in this case as well, the organism was not isolated and studied in pure culture at that time. It is now definitely established that the causative agent of actinomycosis is an anaerobe. This was not fully recognized at first, and subsequent isolations of aerobic cultures, many of them air contaminants, tended to confuse the identity of the organism. The use of the generic name *Actinomyces* was finally limited to a group of anaerobic actinomycetes. This limitation was first sponsored emphatically by Wright, who suggested that this name be used only in connection with the organism causing the disease actinomycosis. All other actinomycetes were placed by him in the genus *Nocardia*. There were various valid reasons for this. He emphasized this as follows: "because the use of the generic term *Actinomyces* for them logically leads to giving the name actinomycosis to those cases of suppurative processes due to infection with certain members of the group."

In 1878, Rivolta suggested the name *Discomyces* for the causative agent of a disease, known as botryomycosis, and now recognized to be due to a true bacterium. The organism was considered, however, to be an actinomyces. This name came into limited use (Merrill and Wade), but was later discarded.

The name *Nocardia* was introduced in 1889 by Trevisan, who considered the generic name *Actinomyces* as untenable because the name *Actinomyce* (without the terminal "s") was given by Meyen in 1827 to a true fungus, *Actinomyce horkelii*. However, according to the International Rules of

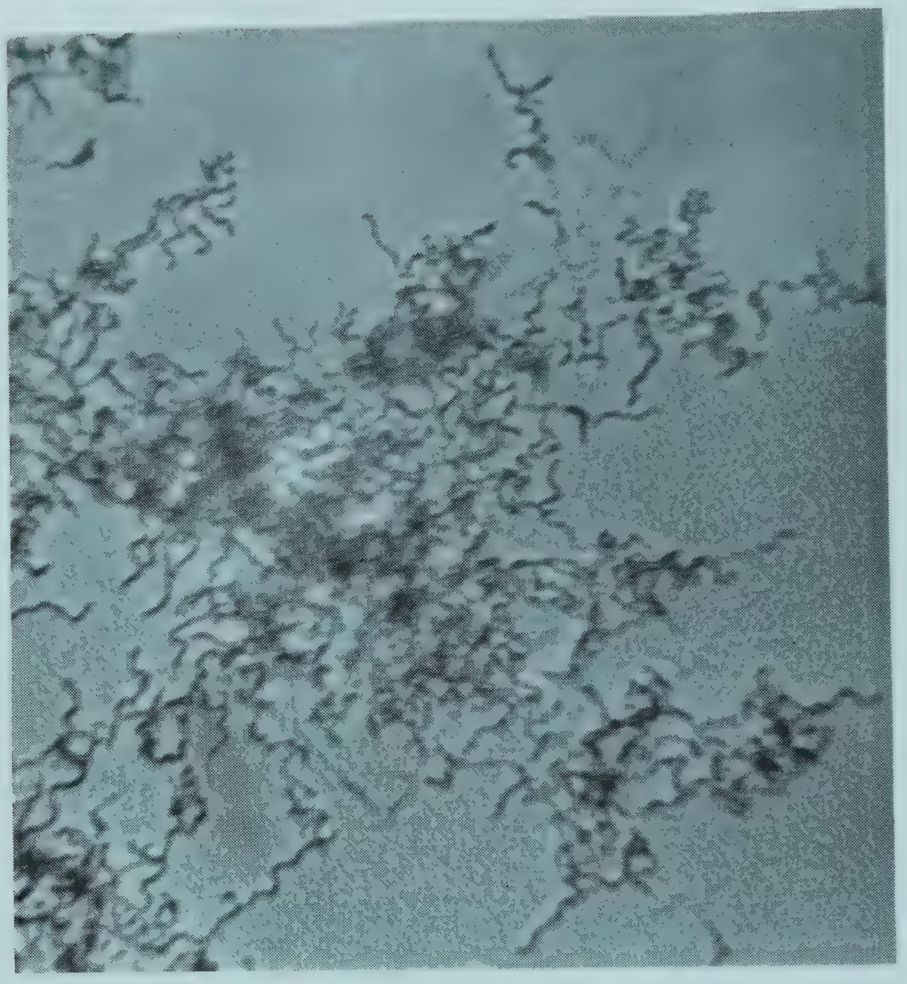


FIGURE 20. Typical growth of a member of the genus *Nocardia*.

Nomenclature, two generic names, if differing even by one letter, are to be regarded as distinct. It was finally decided that the generic name *Nocardia* should not be applied to the group of actinomycetes as a whole, as suggested by Trevisan, but should be limited to only one of the genera of the group, as will be shown later.

Sauvageau and Radais, in 1892, placed the actinomycetes among the true fungi, namely, the Hyphomycetes in the genus *Oospora*. Only a year before, Thaxter (1891) described the causative agent of potato scab, which is a true actinomycete, as *Oospora scabies*. Thaxter himself, however, used this name only provisionally, since he expressed doubt as to the correctness of this designation. *Oospora*, as described by Saccardo, is now recognized as standing for a true fungus. Güssow, who analyzed this name in detail, looked upon the actinomycetes as belonging to the filamentous "higher bacteria," or the Chlamydobacteriaceae; he remarked quite correctly that, "On endeavoring to place the organism in its proper genus, we found ourselves confronted by one of the most perplexing problems of botanical nomenclature, which promises a rich harvest to those who are fond of such study." Even prior to that, namely in 1898, Lachner-Sandoval pointed out the marked morphological differences between *Oospora* and the actinomycetes.

In 1897, Migula considered the name *Cladothrix* Cohn as a proper designation for the actinomycetes. However, the cladothrix group includes certain higher bacteria (*C. dichotoma*) definitely distinct from the actinomycetes; it represents organisms consisting of chains of cells surrounded by a sheath and showing false branching. Earlier, Eppinger (1891) discovered an acid-fast actinomycete, which he believed to exhibit false branching; he designated the organism as *Cladothrix asteroides*, thus introducing another source of confusion in nomenclature.

Petruschky (1913) attempted to recognize both *Actinomyces* and *Streptothrix* as distinct genera, based upon the pathogenicity of the former and the formation of aerial mycelium by the latter. Krainsky (1914) did not consider this difference sufficient to justify the creation of two separate genera; he considered the actinomycetes to be closely related to the mycobacteria. Lehmann and Neumann (1912), however, suggested placing the actinomycetes, as an independent group, between the hyphomycetes and the schizomycetes.

Wollenweber (1921) suggested division of the genus *Actinomyces* into two subgenera: *Pionnothrix*, comprising the forms lacking aerial mycelium and *Aerothrix*, forming a true aerial mycelium. The name *Mycococcus* was proposed by Bokor, in 1930, for an aerial-mycelium-producing actinomycete that was able to decompose cellulose. Neither the biochemical characteristics of the culture nor its morphology justified the creation of this new genus.

In 1936, Brumpt placed the actinomycetes, under the name, *Microsiphonales*, among the Hyphomycetes. The forms parasitic to man were classified as one genus, *Actinomyces* = *Nocardia*.

In addition to the above names, various others were suggested as generic designations for the actinomycetes as a whole or for some of its constituent groups. Among them were *Conidiomyces* Perroncito (1875), *Actinocladothrix* Afanassiev (1889), *Micromyces* Gruber (1891), *Actinobacillus* Lignières and Spitz (1904), *Actinobacterium* Haas (1906), *Cohnistreptothrix* Pinoy (1911), *Actinococcus* Beijerinck (1914), *Anaeromyces* Castellani *et al.* (1921), *Euactinomyces* Langeron (1922), *Brevistreptothrix* Lignières (1924), *Proactinomyces* Jensen (1931), *Asteroides* Puntoni and Leonardi (1935), and a number of others, such as *Actinophyta*, *Indiella*, and *Indiellopsis*. More recently, various additional generic names, such as *Micromonospora*, *Streptomyces*, *Thermoactinomyces*, *Actinoplanes*, *Streptosporangium*, *Microbispora*, *Chainia*, *Waksmania*, *Thermomonospora*, *Thermopolyspora*, were suggested.

Recognition of Species of Actinomycetes

Even greater problems in the classification of actinomycetes appeared in the recognition of specific names, for two important and obvious reasons: 1. No genera can be established without the proper recognition of species. 2. There are so many more species than genera. H. J. Conn said, in 1917, that "no species described in the past can be regarded as characterized sufficiently for recognition except those that are said to be the cause of some definite disease." This becomes particularly evident when one considers such forms as *Actinomyces chromogenus* Gasperini, one of the more readily recognized saprophytic forms. Its characteristic property is the production of a brown pigment on gelatin or peptone media, a property ascribed by Beijerinck to the production of a quinone. In 1914, Krainsky reported four distinct organisms that agreed fully with the original description of Gasperini. Conn mentioned at least 30 forms the published descriptions of which agreed with the *A. chromogenus*. The conclusion was reached that this name should not be recognized even for a group of organisms sufficiently different from other groups.

Similar confusion was found to exist as regards other "species" described earlier, such as *Actinomyces albus* or *A. odorifer*. Descriptions of these organisms were based on the white color of the aerial mycelium or on the formation of an odor, both being properties characteristic of many distinct forms of actinomycetes. As a result, none of the earlier identifications can now be sufficiently recognized.

Sanfelice emphasized, in 1904, the lack of constancy of actinomycete characters when the organisms are grown on ordinary culture media. He abandoned the species concept altogether and suggested establishment of three groups of actinomycetes, each centering around a type species. The groups thus recognized were *A. flavus*, *A. albus*, and *A. violaceus*. Unfortunately, the complex organic media used by Sanfelice did not permit the proper comparisons even of the salient features of the specific organisms. He recognized, however, the value of the pigmentation in classifying actinomycetes, a fact supported by numerous subsequent investigators (Conn and Conn, 1941).

Another 10 years elapsed before the work of Krainsky (1914), on the one hand, and of Waksman and Curtis (1916), on the other, which recognized the unsatisfactory condition of using the common complex organic bacteriological media for characterizing actinomycetes. Krainsky's important contribution to the study of actinomycetes was the introduction of simple synthetic media. He classified the 18 species that he described on the basis of three types of chromogenesis: (a) organisms that secrete soluble pigments into the medium; (b) organisms that produce insoluble pigments, only the colony being colored; (c) those organisms that form a pigmented aerial mycelium. When grown on proper media, each species showed characteristic properties, based upon the above three types of pigmentation. These properties are now universally recognized, in addition to morphological and certain biochemical characteristics, as among the most essential in establishing species of actinomycetes. Krainsky further divided his cultures into two groups, on the basis of the size of the colony: (a) a macrogroup producing large colonies with oval or spherical conidia; (b) a microgroup producing small colonies and spherical conidia only. Unfortunately, however, he did not study the morphology of the aerial mycelium.

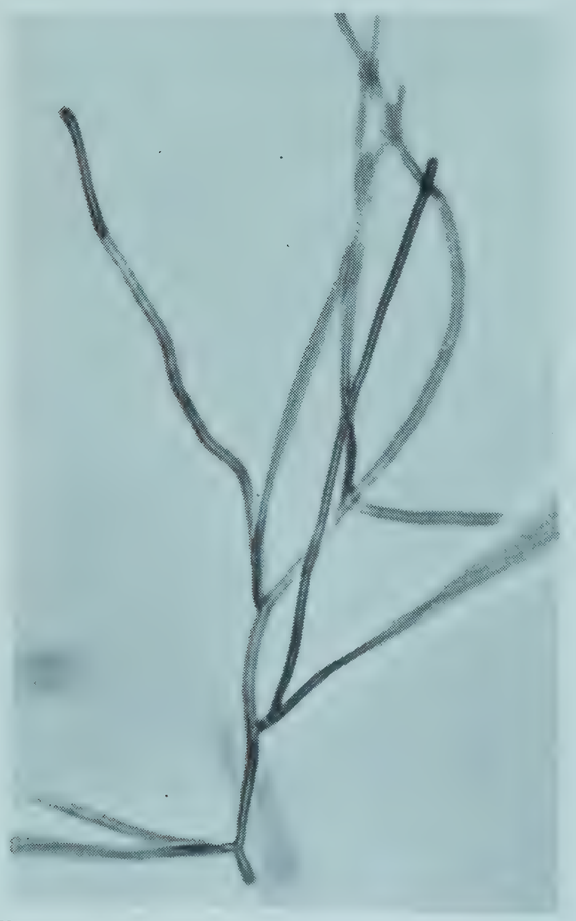


FIGURE 21. Growth of a streptomyces producing straight sporophores.

Soon afterward, Waksman and Curtis published descriptions of 18 additional species. Their classification was also based upon pigmentation of the cultures in synthetic and organic media and upon certain cultural and morphological properties, such as liquefaction of gelatin. They disregarded the size of the colonies and emphasized spiral formation in the aerial mycelium as characteristic criteria in species differentiation. Waksman (1919) further recognized the importance of the group concept in characterizing and classifying actinomycetes. With the rapid increase in the number of new species described, this concept recently has been gaining ever greater recognition.

This brief review of the early attempts at nomenclature and classification of actinomycetes brought out the fact that two generic names, *Streptothrix* and *Actinomyces*, have always occupied a prominent place. To establish the present position of these two generic names, and especially their bearing upon subsequent systems of classification, their historical significance may be analyzed in further detail.

Streptothrix

The name *Streptothrix* was proposed by Cohn for an organism belonging to the thread-forming bacteria and “producing concretions in the lachrymal duct.” It was described later by De Toni and Trevisan (1889) as “filamentis tenuissimis, hyalinis, parallele insimul stratiformi-coalitis vel fasciculatis, rectis vel incurvis, sparse irregulariterque ramosis, in fragmenta inaequalia secedentius.”

As has been pointed out, this name became the cause of considerable confusion, due, on the one hand, to the fact that it had been used previously by Corda for one of the Hyphomycetes and had been codified as a true fungus by Saccardo in his Sylloge Fungorum; on the other hand, Cohn himself did not differentiate the organism sufficiently from *Cladothrix*, which produced false branching (see also Macé). Frequent confusion with many other names given to the group, notably *Oospora*, *Actinomyces*, and *Nocardia*, did not contribute toward firm establishment of the name *Streptothrix* in the literature on the actinomycetes. Most of those who used this generic name, beginning with Gasperini in 1889 and ending with Gratia and Dath in 1924, included in it the air-borne forms, and especially the forms producing aerial mycelium and true spores. This can be seen from the description of *Streptothrix* by Chester (1901): “Cells in their ordinary form as long branched filaments. Cultures on solid media raised. Growth coherent, dry, rough or crumpled, often with a moldy appearance due to the formation of aerial hyphae. Without endospores, but by a multiple segmentation of a filament, the production of short, conidia-like bodies.”

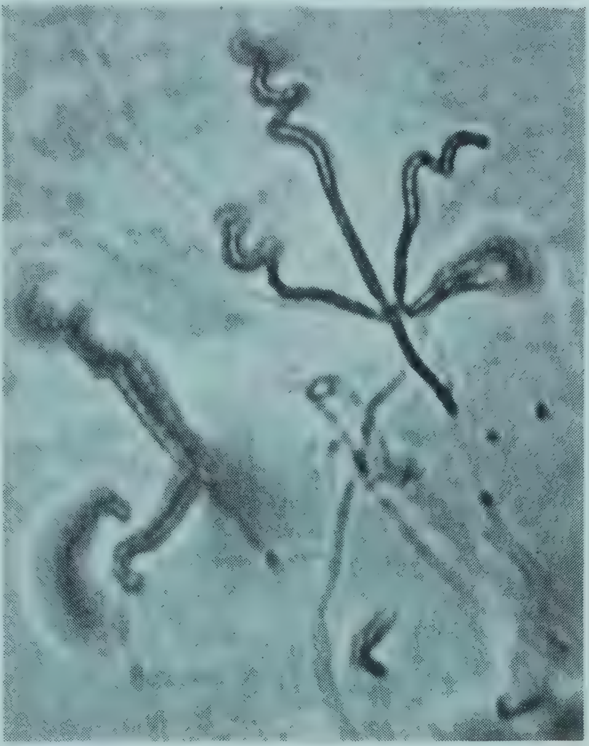


FIGURE 22. Growth of a spiral producing streptomyces.

Various investigators, notably Rossi-Doria, Foulerton, and Musgrave and Clegg, and others adopted the name *Streptothrix* largely for the reason that a better generic designation for certain organisms was lacking. Caminiti (1907) listed as many as 41 species, under this generic name. These included a highly confusing conglomerate, ranging from *Streptothrix actinomyces* Rossi-Doria, which he considered as identical with *Actinomyces bovis* Harz, to *Streptothrix mihi* Caminiti, and from *St. eppingeri* (*Cladothrix asteroides*), of which *St. aurantiaca* was considered as a nonpathogenic form, through *St. rubra* or *madurae* of Vincent, to *St. erysipeloides* Rosenbach.

On the other hand, some of the taxonomists like Lehmann and Neumann and E. F. Smith discarded the name *Streptothrix* completely. Rullmann (1917) also emphasized the lack of validity for this name. Buchanan (1925) finally considered the name *Strepto-*

thrix as a generic designation among the bacteria as invalid.

We hoped that Buchanan's final decision would stand. Unfortunately, this was not the case. As recently as 1936, Woytek still considered *Streptothrix* as the proper designation for the anaerobic pathogens and still reserved the name *Actinomyces* for the aerobic saprophytes. Erikson (1940) was most emphatic in directing attention to this historical misinterpretation.

Actinomyces

The name *Actinomyces* is the most common one used to designate a group of actinomycetes. As pointed out above, it was first used by Harz for a genus of thread-forming bacteria observed in the pus of cattle affected by "lumpy jaw." The pus consisted of granules made up of "slender filaments, irregularly branched, radiating from the center, and with the ends of the filaments in the form of refractive swellings." The Greek word *Actinomyces* means ray-fungus. Harz never grew this organism in pure culture, but he noted that it is the causative agent of actinomycotic infections in animals.

Various subsequent investigators adopted this name for the actinomycetes as a whole. Trevisan, however, recognized the relationship between organisms described as *Streptothrix* and *Actinomyces*, and suggested discarding both these names in favor of *Nocardia*. Among those who used the name *Actinomyces* for actinomycetes as a whole, should be mentioned Gasperini (1895), Lachner-Sandoval (1898), Levy (1899), Berestnew (1899), and later Lehmann and Neumann (in the editions of their book subsequent to 1896), Orla-Jensen, Waksman (in his early work and in the early editions of the Bergey Manual), and Krassilnikov.

Stokes (1904) examined the various generic names proposed for the pathogenic actinomycetes and came to the conclusion that the name *Actinomyces* should be used, since all the others had been preempted. He classified the genus into seven species: *A. bovis*, *A. asteroides*, *A. israeli*, *A. nocardi*, *A. madurae*, *A. caprae*, *A. vesicae*.

As pointed out above, Wright proposed limiting the name *Actinomyces* to the parasitic forms which produce rays in tissues; he used the name *Nocardia* for the forms producing aerial mycelium and spores. The same concept was adopted by Jordan. Petruschky accepted the separation of the actinomycetes into two groups, although he preferred the name *Streptothrix* to that of *Nocardia* for the second group. Pinoy also divided the actinomycetes into two groups: *Nocardia* comprising the aerobic, spore-producing forms, and *Cohnistreptothrix* (rather than *Actinomyces*) for the anaerobic, nonsporulating forms.

Dresel (1925) finally disposed completely of the confusion between (a) the anaerobic organism, as clearly elucidated by the work of Wolff-Israel, causing actinomycosis and producing sulfur granules, for which the generic name *Actinomyces* Harz must be retained, and (b) the aerobic organisms, comprising some that are widely distributed in nature as saprophytes and others that are able to cause infections. He stated emphatically that if the name *Streptothrix* cannot be retained for the second group, clearly a new name must be found. This lack of recognition of the distinction among the anaerobes and aerobes has been responsible for much of the confusion in the designation and classification of actinomycetes, beginning with Bostroem's isolation from an actinomycotic infection of a contaminating aerobe and continuing through the work of numerous subsequent investigators. These included such eminent contributors to the subject as Lieske, who kept emphasizing the change of the anaerobe into an aerobic form, and Krassilnikov, who refused to conceive of purely anaerobic forms, in spite of the impressive and clear-cut evidence to that effect.

In time, however, the generic name *Actinomyces* came to be recognized for the anaerobic group of actinomycetes concerned with the causation of human and animal actinomycosis accompanied by the formation of granules.

Gradually, the position of the actinomycetes as a biological system became recognized. Before proceeding with the characterization of the actinomycetes as an independent group of microorganisms and with their proper classification into genera and species, we must consider their relationship to two other groups of microorganisms, namely, the true bacteria and the true fungi.

Relation of Actinomycetes to Bacteria and Fungi

F. Cohn considered his streptothrix as a bacterial form. Almquist (1890) also looked upon this group of organisms as belonging to the bacteria, in spite of Brefeld's assertion that they are closely related to the filamentous fungi. Almquist himself isolated in pure culture three organisms, one as a dust contaminant, another from water, and a third from the body of a person who recently had died from cerebrospinal meningitis. All appeared to be forms that would now be considered as members of the genus *Streptomyces*, although the third form did not produce any aerial mycelium.

Since then, considerable discussion has taken place concerning the relationship of the actinomycetes to the bacteria, on the one hand, and the fungi, on the other. The information that has been gradually accumulating tends to emphasize that actually the actinomycetes are more closely related to the true bacteria.

Relationship to bacteria. The following facts may be presented to substantiate this relationship:

1. The diameters of the hyphae and spores of actinomycetes (usually 0.5–1.0 μ) are similar to those of bacteria but not of fungi.

2. Many actinomycetes reproduce by fragments or oidia that are similar in size and in shape to the rod-shaped and spherical bacteria.

3. Many actinomycetes produce no true aerial mycelium; their growth appears similar to that of certain pleomorphic bacteria, especially to corynebacteria.

4. Many actinomycetes are acid-fast; they resemble, both in their morphology and in their physiology, the mycobacteria. This is true particularly of members of the genus *Nocardia*.

5. Nuclear staining, phase microscopy, and vital staining with diachromes and fluorochromes show that actinomycetes possess nucleoids. No true mitosis could be demonstrated, nor any nucleus by means of the Feulgen reaction. All these facts suggest the close systematic relationship of the actinomycetes to the bacteria.

6. The chemical composition of the cells points to the relationship of the actinomycetes to the bacteria rather than to the fungi. When the spores are liberated as a result of the break-up of the sporophores, the empty shells become visible; the cell wall is soluble in 10 per cent KOH solution and in antiformin. When the cells of *Streptomyces* were treated with lysozyme, the cell walls were found to be mucoid in nature and were hydrolyzed to glucosamine.

7. Cells of actinomycetes do not contain chitin or cellulose, characteristic of true fungi.

8. Actinomycetes, like true bacteria, are procaryotes, whereas fungi are eucaryotes.

9. The phenomena of lysis and phage sensitivity among the actinomycetes point further to their relationship to the bacteria rather than to the fungi.

10. Finally, the recent evidence concerning the sensitivity of the actinomycetes to antibiotics places them definitely with the bacteria and not with the true fungi.

These facts fully justify the conclusion

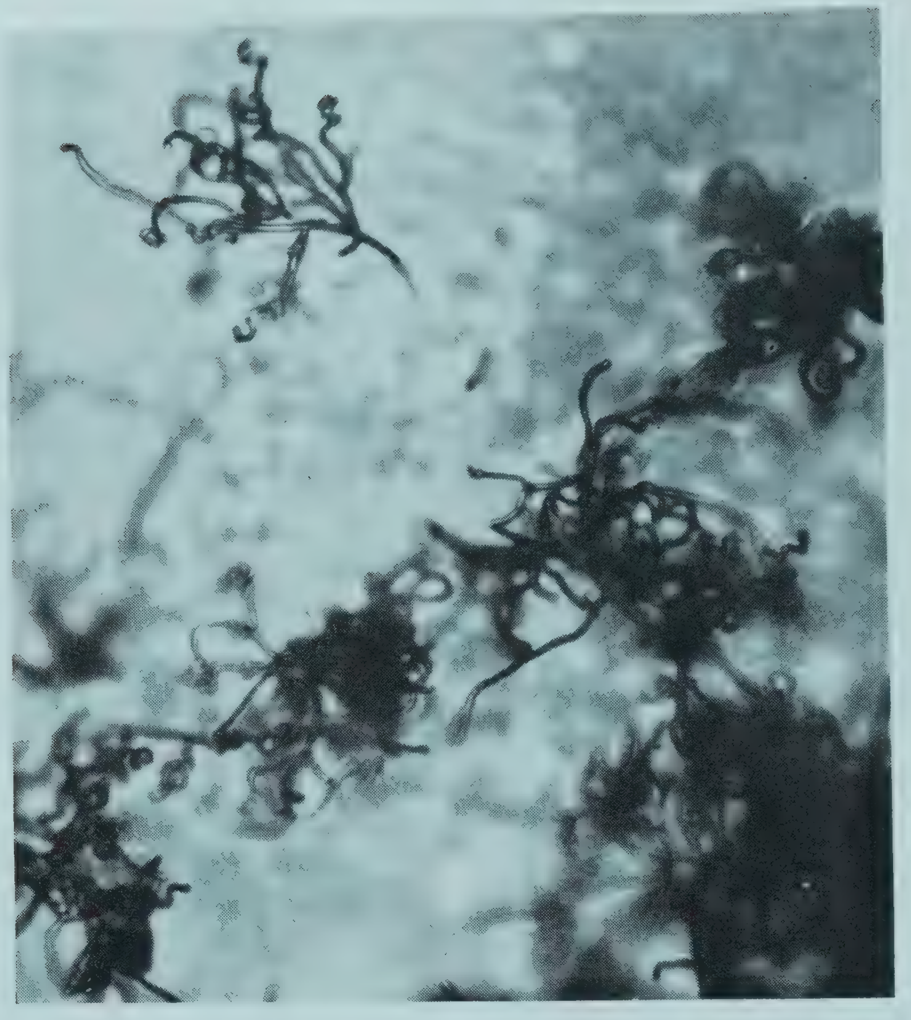


FIGURE 23. *S. fradiae*, another spiral producing organism.

that the actinomycetes should be classified with the bacteria, under the Schizomycetes. No wonder that Afanassiev suggested in 1889 that the filament of an actinomycete is a gigantically elongated bacterial cell. Among the other earlier investigators, the following considered actinomycetes as bacteria: Cohn, Bostroem, Wolff and Israel, Berestnew, and Lieske. Various subsequent investigators emphasized the particular relationship of certain actinomycetes, notably the *Nocardia* group, to the mycobacteria.

Grootten, for example, (1934) analyzed the relationship between the actinomycetes and the hyphomycetes, on the one hand, and the bacteria, on the other. He emphasized that the fact that actinomycetes are often branched does not justify in itself their classification with the fungi, since certain bacteria (*Mycobacterium*, *Corynebacterium*, etc.) can, under certain conditions, give branching forms. Further, the cytological structure of the actinomycetes, characterized by the absence of well-defined morphological nuclei, definitely places them with the bacteria. He concluded that the Actinomycetales are closely related to the Mycobacteriales, as characterized by the evidence of bacillary and filamentous forms taking the Gram stain, sometimes by acid resistance, and by capability of branching.

Relationship to fungi. The relation of the actinomycetes to fungi, especially the Fungi Imperfecti, was based upon the following similarities:

1. The production and manner of branching of the aerial mycelium and the manner of spore formation, especially in the genera *Streptomyces*, *Micromonospora*, *Waksmania*.

Streptosporangium, and *Thermopolyspora*, appear to resemble those of fungi.

2. The nature of the growth of many actinomycetes on the surface of liquid and solid media and their growth in a suspended or submerged condition are similar to those of fungi. Stationary liquid cultures of actinomycetes do not usually show turbidity. In this, they are similar to the fungi.

3. Pleomorphism of certain actinomycetes shows similarity to that of fungi (Grigorakis).

4. Roach and Silvey believed that the evidence for the origin of the secondary mycelium lends credence to the theories of those investigators who believe actinomycetes are true fungi.

An early student of the morphology of actinomycetes, Domec, concluded that these organisms must definitely be removed from the bacteria and placed with the fungi. Vuillemin (1931), who considered the causative agent of "farcin du boeuf" as a species of *Nocardia* (*N. farcinica*), also believed that the group as a whole belongs to the fungi, in the family Microsiphonales, in the order *Arthrospora*. The mycobacteria were also placed in this family. Among the earlier investigators who classified the actinomycetes with the fungi were Harz, Gasperini, Sauvageau and Radais. More recently, Pridham *et al.* (1958), on the basis of their morphological studies, were also inclined to classify the actinomycetes, especially the genus *Streptomyces*, with the fungi.

Transition group. Because of these apparently conflicting facts, the suggestion has been made that actinomycetes should be placed in a taxonomical transition group between the Schizomycetes and the Hyphomycetes. Many highly heterogeneous properties of some of the actinomycetes, especially those of a morphological nature, have further strengthened the idea of placing them between the true bacteria and the true fungi.

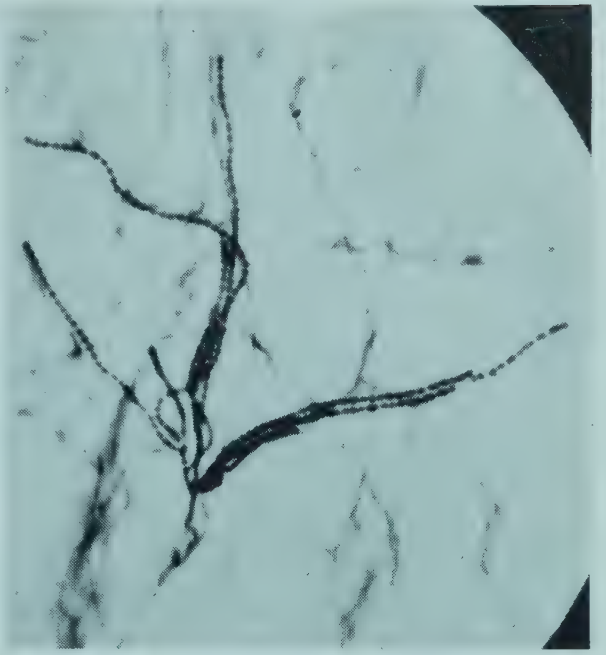


FIGURE 24. Broom-shaped sporophores of a streptomyces.

Among the investigators who held to the middle position, one should mention Rossi-Doria, Kruse, Claypole, Lieske (in his later work, 1928), Waksman (especially in his earlier work), and Krassilnikov. Krassilnikov looked upon the actinomycetes as an independent group of microorganisms with its own typical morphological, cultural and physiological properties.

Classification Systems of Actinomycetes

Numerous systems have been proposed for classifying actinomycetes. These were based upon the causation of disease and the ecology of the organisms, their morphology (structure of sporophores, shape of spores), cultural activities (growth on various media), physiological (oxygen tension, temperature optima) and biochemical (enzymatic mechanisms, sugar utilization, acid production) properties, serum diagnosis, and formation of soluble and insoluble pigments.

Buchanan made a comprehensive review of the various systems of bacterial classification, in which the natural position of the bacteria (beginning with Mueller's classification of *Vermes* in 1773) was outlined, as well as the position of the actinomycetes among the bacteria (utilizing Cohn's system of

1875). Some of the more significant systems, in which the actinomycetes were given important consideration, may be summarized here.

Earlier Systems of Classification

Migula (1894) placed the actinomycetes (*Streptothrix* Cohn emend) under the *Chlamydobacteriaceae.* Lehmann and Neumann (1896) included the actinomycetes among the bacteria-like Hyphomycetes, which were separated from the true bacteria. They considered three genera at first: *Corynebacterium* Lehmann and Neumann, *Mycobacterium* Lehmann and Neumann, and *Oospora* Wallroth. The last comprised the forms which gave "Mycelium filaments long, often bent, without sheath, with true branching. Many species produce conidia on aerial hyphae. Not acid-fast." Chester (1897) created a new order, *Mycobacteriaceae* in which he included *Corynebacterium* and *Mycobacterium*, and, like Migula, placed the actinomycetes under the name *Streptothrix* among the *Chlamydobacteriaceae.*

In 1899, Chester revised his classification and included *Streptothrix* among the *Mycobacteriaceae.* The genus was described as follows: "Cells in their ordinary form as long branched filaments. Produce conidia-like bodies. Cultures generally have a mouldy appearance due to the development of aerial hyphae."

Schahad (1904) divided the actinomycetes into two groups: *Actinomyces typica*, with thickened mycelium, and *Actinomyces atypica*, with nonthickened mycelium.

Jordan, in 1908, divided the thread-forming bacteria into four genera under Trichomycetes:

1. Filaments unbranched—*Leptothrix*
2. Filaments with pseudomembranes—*Cladothrix*
3. Filaments with true branches:
    a. Reproductive elements, spores observed—*Nocardia*
    b. No spores observed—*Actinomyces*

H. W. Conn, in his *Agricultural Bacteriology* (1909), divided the higher bacteria into four genera: *Cladothrix*, *Leptothrix*, *Streptothrix*, and *Actinomyces.* Orla-Jensen (1909) created a family—*Actinomycetes* in the order Cephalotrichinae. This family was divided into four genera: *Rhizomonas* (nodule-forming bacteria), *Corynemonas* (parasites, nonacid-fast), *Mycomonas* (parasites, acid-fast), *Actinomyces* (much branching mycelium).

Engler (1912) placed the actinomycetes among the Eubacteria, in the family *Actinomycetaceae*, under one genus *Actinomyces*: "Filamentous colonies with true branching; radiating, nonmotile. Filaments dividing into oidia."

Chalmers and Christopherson (1916) proposed a system of classification based upon the pigmentation of the granules in the infected material.

1. Actinomycotic granules black, not cultivated on artificial media.
2. Granules yellow, orange, red, or colorless, divided into two species:
    a. *Cohnistreptothrix*—anaerobes, cultivated with difficulty.
    b. *Nocardia*—aerobes, cultivated readily.

Krainsky divided the actinomycetes, on the basis of colony size, into *Macroactinomyces* and *Microactinomyces.*

Buchanan (1917, 1918) created a new order Actinomycetales, which included a single family, *Actinomycetaceae.* This family was divided into four genera, two of which—*Actinomyces* and *Nocardia*—are most pertinent to this treatment. This system, because of its historical significance is given here in detail.

Actinomycetales

Syn. *Actinomycetes* Balbiani, *Trichobacteriaceae* Fischer

Mold-like organisms, not typically water forms, saprophytic or parasitic. Sheath not impregnated with iron, true hyphae with branching often evident, conidia may be developed, but never endospores. Without granules of free sulfur and without bacteriopurpurin. Never producing a pseudoplasmodium. Always nonmotile.

The order Actinomycetales contains a single family, *Actinomycetaceae*.

Family I. *Actinomycetaceae*

A. No evident aerial threads or conidia formed. Usually parasitic. Often anaerobic or microaerophilic.
  1. Threads usually not branched.
    Threads disjointing very readily; long mycelial threads uncommon. Genus 1. *Actinobacillus*
    b. Threads longer, not disjointing into short rods .... Genus 2. *Leptotrichia*
  2. Threads more or less branched, frequently clubbed in tissues..... Genus 3. *Actinomyces*
B. Aerial threads and conidia evident on culture media..................... Genus 4. *Nocardia*

Genus 3. *Actinomyces* Harz

Branched filaments, resembling mycelium, breaking up into segments which may function as conidia. Usually parasitic. Clubbed ends conspicuous in lesions. Not producing aerial hyphae or conidia.

The type species is *Actinomyces bovis* Harz, the cause of bovine actinomycosis.

Genus 4. *Nocardia* Trevisan

Branched filaments, resembling a mycelium, readily breaking up into segments. Usually saprophytic. Aerial threads and conidia commonly produced.

In the preliminary report of the Committee of the Society of American Bacteriologists (Winslow *et al.*, 1917), the genera *Actinomyces* and *Nocardia* were placed in the family *Mycobacteriaceae*, in the order Eubacteriales. The difference between the two genera was based on the assumption that members of the genus *Actinomyces* do not produce any aerial mycelium and are usually parasitic.

Castellani and Chalmers (1919) included the actinomycetes among the Hyphomycetes, in the order Microsiphonales, created by Vuillemin in 1912. This order was divided into two families, 1. *Nocardiaceae*, forming a mycelium (Syn. Actinomycetes Lachner-Sandoval, 1898; Trichomycetes Petruschky, 1903); 2. *Mycobacteriaceae*, without a mycelium.

The *Nocardiaceae* were divided as follows:

I. Grow aerobically, easy to cultivate, and produce arthrospores.
  Genus 1. *Nocardia* De Toni and Trevisan, 1889.

II. Grow best anaerobically, but can often grow aerobically; difficult to cultivate and do not produce arthrospores.
  Genus 2. *Cohnistreptothrix* Pinoy, 1911.

In the final report of the Committee of the Society of American Bacteriologists (Winslow *et al.*, 1920) the family *Mycobacteriaceae* was elevated to the order Actinomycetales, which was divided into two families: *Actinomycetaceae* and *Mycobacteriaceae*. The genus *Actinomyces* was placed in the first family. The genus *Nocardia* was dropped (see also Breed and Conn, 1919).

The following description of the order Actinomycetales was given in the first edition of Bergey (1923, p. 337): "Cells usually elongated, frequently filamentous and with a decided tendency to the development of branches in some genera giving rise to the formation of a definite branched mycelium. Cells frequently show swellings, clubbed or irregular shapes. Some species are parasitic in animals or plants. As a rule strongly aerobic (except for some species of *Actinomyces* and the genera *Fusiformis* and *Leptotrichia*) and oxidative. Growth on culture media often slow; some genera show moldlike colonies." The order was divided into two families: 1. The *Actinomycetaceae*, containing, in addition to the first three genera included by Buchanan (the genus *Nocardia* having been dropped), also the genus *Erysipelothrix*. 2. The *Mycobacteriaceae* with three genera: *Proactinomyces*, *Corynebacterium*, and *Mycobacterium*.

Lehmann and Neumann, in their later editions, divided the actinomycetes into two families: *Proactinomycetaceae* with two

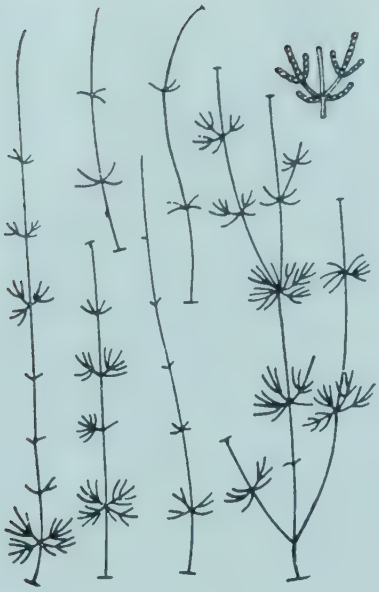


FIGURE 25. *S. rubrireticuli*, a verticil-forming streptomyces (Reproduced from: Shinobu, R. Mem. Osaka Univ. No. **4B**: 74, 1955).

genera, *Corynebacterium* and *Mycobacterium*; *Actinomycetaceae*, with only one genus *Actinomyces*. Kluyver and van Niel suggested the removal of the family *Mycobacteriaceae* from the Actinomycetales altogether.

Langeron suggested the following system of classification of actinomycetes:

I. *Euactinomyces*, aerobes.

II. *Cohnistreptothrix*, anaerobes, grow poorly on artificial media.

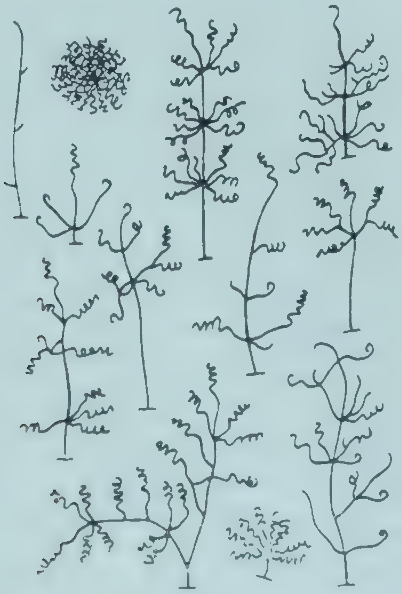


FIGURE 26. Anitella type verticil-forming streptomyces (Reproduced from: Shinobu, R. Mem. Osaka Univ. No. **4B**: 75, 1955).

Recent Systems of Classification

Ørskov, in 1923, divided the actinomycetes into three genera:

1. *Cohnistreptothrix*, nonseptate mycelium, forming readily sporulating aerial hyphae.

2. *Actinomyces*, mycelium septated:
   a. Cultures forming aerial mycelium.
   b. Cultures not forming any aerial mycelium.

3. *Micromonospora*, with single spores borne at the ends of branches.

Jensen (1931) modified the above system by retaining the last genus unchanged, and by designating the first genus as *Actinomyces* and the second as *Proactinomyces*, as shown here:

A. No spores are formed.
Family *Proactinomycetaceae*
    I. No mycelium is formed.
        a. Acid-fast organisms.
        Genus *Mycobacterium*
        b. Nonacid-fast organisms.
        Genus *Corynebacterium*
    II. Mycelium is formed.
    Genus *Proactinomyces*
B. Spores are formed.
Family *Actinomycetaceae*
    I. Spores in aerial mycelium.
    Genus *Actinomyces*
    II. Spores terminally on branches of vegetative mycelium
    Genus *Micromonospora*

Lignières (1922) divided the actinomycetes into three groups:

1. *Actinomyces*. Aerobes; mycelial hyphae long, not breaking up into rods.

2. *Brevistreptothrix*. Anaerobes; mycelial hyphae short, break up into long rods.

3. *Actinobacillus*. No mycelium. Cells rod-shaped.

Puntoni and Leonardi (1935) divided the actinomycetes into three genera:

1. *Actinomyces* Harz, aerobic, producing aerial mycelium and arthroconidia.

2. *Actinobacterium* Haas (*Cohnistrepto-*

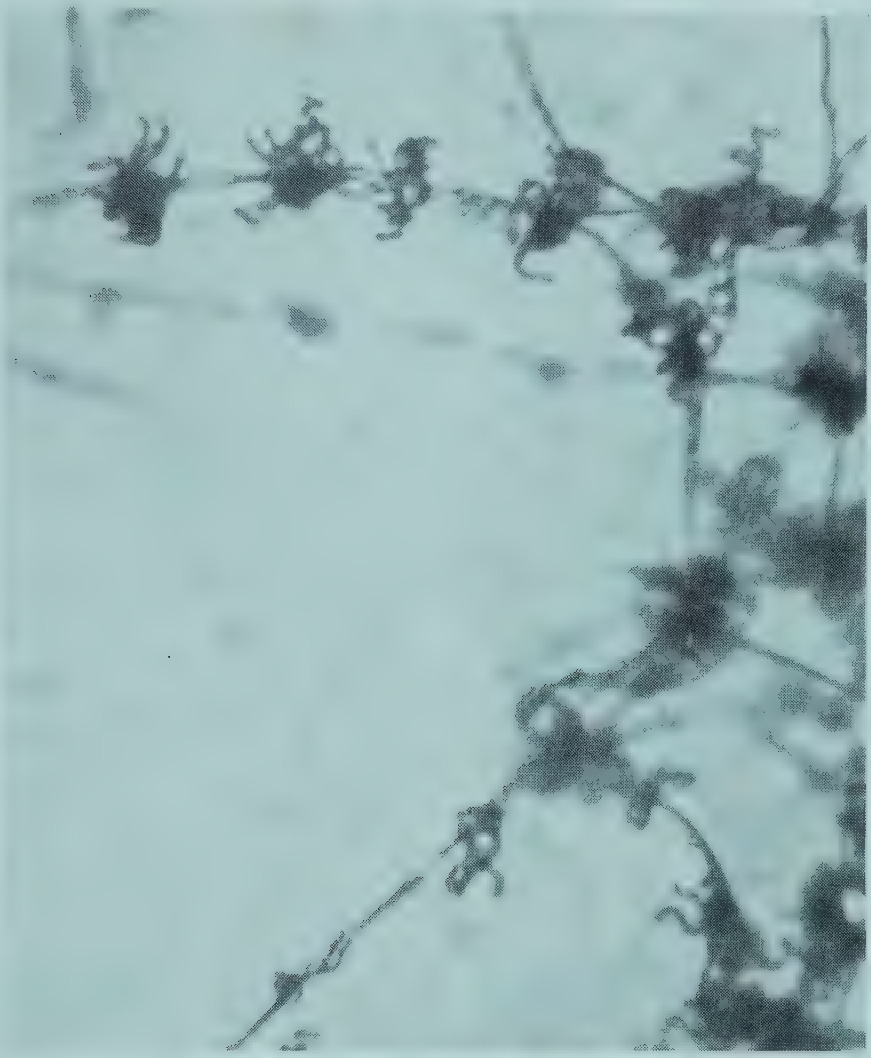


FIGURE 27. *S. rimosus.*

thrix Pinoy), anaerobic, without aerial hyphae.

3. *Asteroides*, aerobic, without aerial mycelium, fragmenting into bacillary fractions, partially acid-fast, little adherent colonies.

These three genera correspond fairly closely to the presently recognized *Streptomyces*, *Actinomyces*, and *Nocardia*, respectively.

In 1939, there were presented before the Third International Congress for Microbiology, a series of papers on the classification of actinomycetes that can be briefly summarized here.

Naeslund (1940) emphasized that the following characteristics should be considered: (1) branching or nonbranching of the hyphae; (2) filamentous, mycelial forms or isolated rod-shaped elements; (3) wavy or straight filaments; (4) presence or absence of conidial spores; (5) aerobic or anaerobic manner of growth; (6) saprophytic or "animal-pathogenic" forms. No importance was attached to club-formation, presence of radiate granules, acid-fast character, presence of "wavy" filaments or dissimilarities in metabolism. Puntoni (1940) suggested division of the actinomycetes on the basis outlined by Puntoni and Leonardi. Erikson emphasized the justification of distinguishing the two anaerobic forms, namely the "israeli," or human type, and the "bovis," or microaerophilic forms found in cattle. Krassilnikov and Waksman and Umbreit presented their systems which are now discussed in detail.

Krassilnikov at first accepted the classifi-

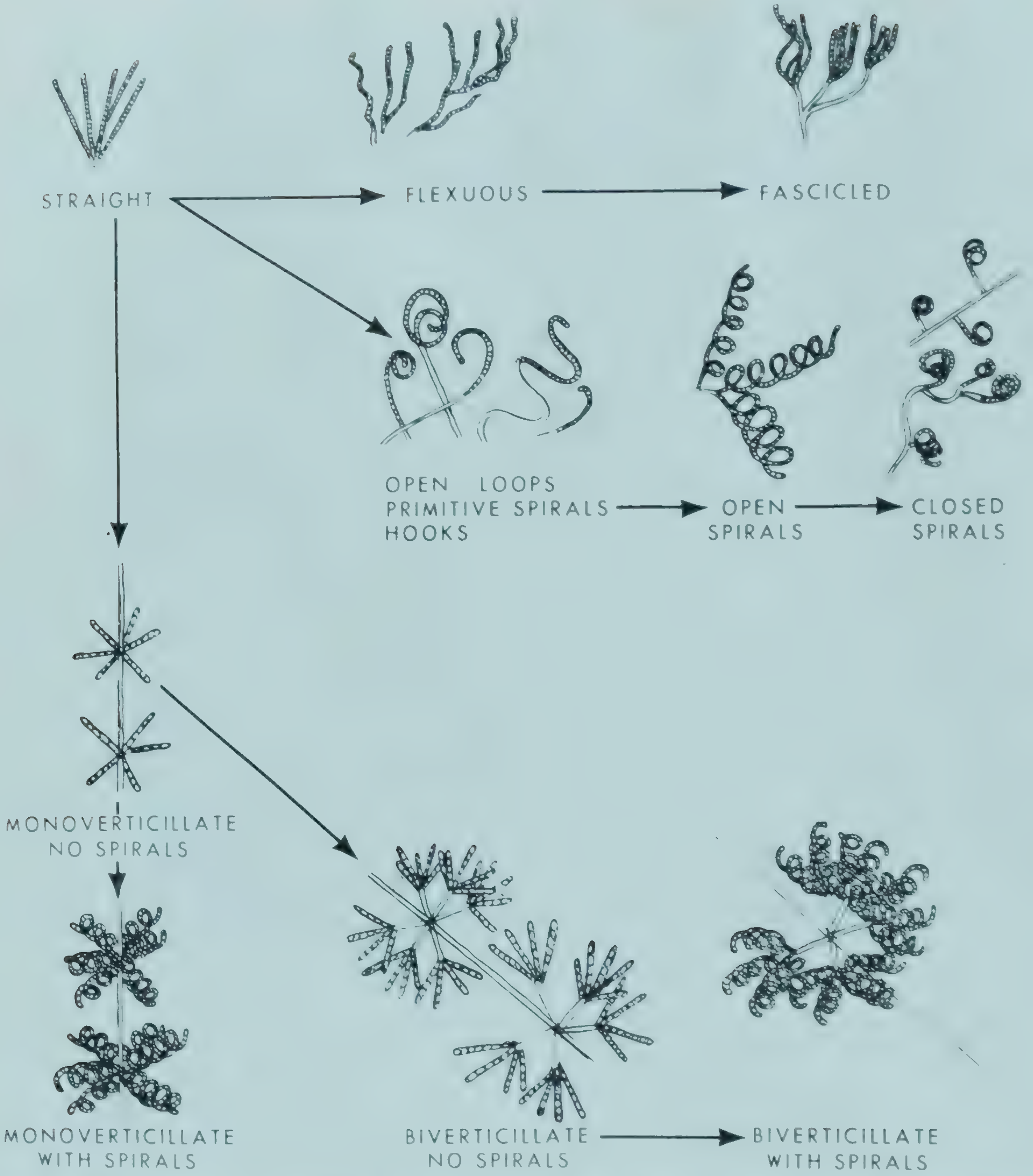

STRAIGHT
FLEXUOUS
FASCICLED
OPEN LOOPS
PRIMITIVE SPIRALS
HOOKS
OPEN SPIRALS
CLOSED SPIRALS
MONOVERTICILLATE NO SPIRALS
MONOVERTICILLATE WITH SPIRALS
BIVERTICILLATE NO SPIRALS
BIVERTICILLATE WITH SPIRALS

FIGURE 28. Suggested evolutionary development of the sporophore structure in the genus *Streptomyces* (Reproduced from: Pridham, T. G. *et al*. Appl. Microbiol. **6**: 54, 1958).

cation proposed for the order Actinomycetales by Buchanan. He divided it into two families: *Actinomycetaceae*, with four genera, *Actinomyces*, *Proactinomyces*, *Mycobacterium*, and *Mycococcus*; and *Micromonosporaceae*, with one genus *Micromonospora*. Later, he suggested (1941, 1949) division of the actinomycetes into three families: *Actinomycetaceae*, with two genera, *Actinomyces* and *Proactinomyces*; *Micromonosporaceae*, with one genus, *Micromonospora*; and *Mycobacteriaceae*, with two genera, *Mycobacterium* and *Mycococcus*.

In 1940, Waksman and Umbreit, and later Waksman, proposed the following system of classification of the Actinomycetales:

A. Mycelium rudimentary or absent: Family *Mycobacteriaceae* Chester.
  I. Acid-fast organisms: *Mycobacterium* L & N
  II. Nonacid-fast organisms: *Corynebacterium* L & N
B. Mycelium produced:
  I. Substrate mycelium divides by segmenta

tion into bacillary or coccoid elements: Family *Proactinomycetaceae* L & N.

1. Anaerobic or microaerophilic, usually parasitic, nonacid-fast organisms: *Cohnistreptothrix* Pinoy.
2. Aerobic, partially acid-fast or nonacid-fast: *Proactinomyces* Jensen. This group is divided into two subgroups:
    - a. Partially acid-fast, nonproteolytic, nondiastatic, utilize paraffin. Usually yellow, pink, or orange to orange-red in color.
    - b. Nonacid-fast, diastatic, largely proteolytic, do not utilize paraffin. Yellow, orange to black in color.

II. Substrate mycelium normally remains undivided:

1. Multiplication by conidia formed in chains from aerial hyphae: Family *Actinomycetaceae* Buchanan: *Actinomyces* Harz. This group is divided into five subgroups:
    - a. Straight sporulating hyphae, monopodial branching, never producing regular spirals.
    - b. Spore-bearing hyphae arranged in clusters.
    - c. Spiral formation in aerial mycelium; long, open spirals.
    - d. Spiral formation in aerial mycelium; short, compact spirals.
    - e. Spore-bearing hyphae arranged on mycelium in whorls or tufts.
2. Multiplication by spores formed terminally and singly on short conidiophores: Family *Micromonosporaceae* Krassilnikov; *Micromonospora* Ørskov. This group is divided into three subgroups:
    - a. Simple spore-bearing hyphae.
    - b. Branching spore-bearing hyphae.
    - c. Spore-bearing hyphae in clusters.

This system, especially the use of the generic name *Cohnistreptothrix* for the causative agent of actinomycosis and of *Actinomyces* for the aerobic forms producing aerial mycelium, was subjected to a certain amount of criticism. It was emphasized that although Harz's description of *Actinomyces bovis* was perhaps vague, there was no question concerning the nature of the disease caused by the organism, and the chances were overwhelmingly in favor of his having actually observed the anaerobic pathogenic filamentous form. Under the Botanical Code, the name *Actinomyces* had, therefore, to be applied either to the organism of "lumpy jaw" or not used at all. It had to be restricted, therefore, to the anaerobic, pathogenic species. A new generic name had to be found for the aerobic, saprophytic, spore-forming species. Waksman and Henrici (1943) gave careful consideration to all the names previously applied to organisms of this type. They came to the conclusion that the great majority of such names were invalid and must be rejected either because they were first used as synonyms of *Actinomyces* or they were previously applied to entirely different types of organisms.

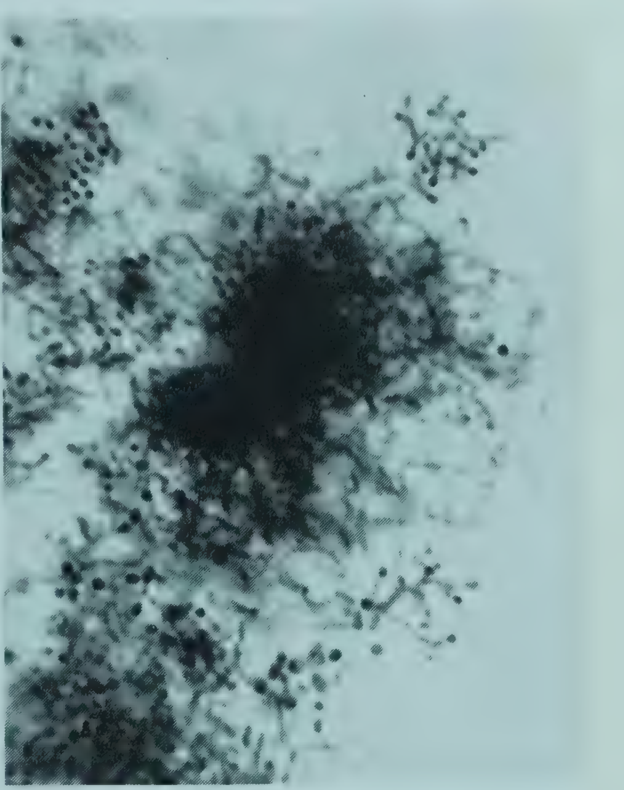


FIGURE 29. Typical growth of *Micromonospora*.

The name *Nocardia* was then given special consideration. After its introduction by Trevisan in 1888, it had been widely used, sometimes for the actinomycetes as a whole; occasionally only the saprophytic aerobic species were included. De Toni and Trevisan placed five species in the genus *Nocardia*, the first of these being *N. farcinica*. This

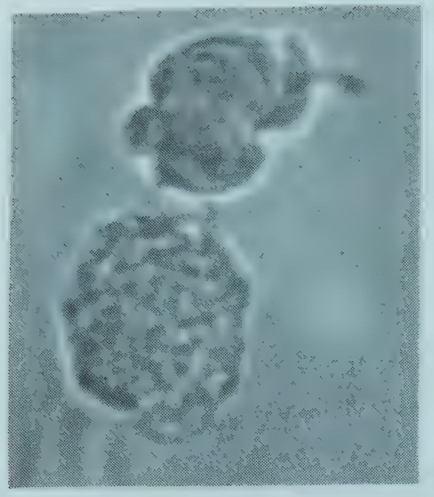


FIGURE 30. The genus *Actinoplanes*

species was described, but not named, by Nocard. Trevisan regarded it as the type species of the new genus. *N. actinomyces* Trevisan (Syn. *Actinomyces bovis* Harz) was given as the second species. This was followed by *N. foersteri* (Cohn) Trevisan (Syn. *Streptothrix foersteri* Cohn). In considering these facts, Breed and Conn concluded that "There appears to be no justification for the use of the term *Nocardia* Trevisan for the entire group of organisms included in the Actinomycetaceae. It may, however, be properly used for a subdivision of the genus *Actinomyces*, provided, however, *N. farcinica* is retained in the genus *Nocardia* and is established as the type of the genus."

Waksman and Henrici further considered the need of a valid name for the aerobic sporulating species. They concluded that the only solution for this problem was to coin a new generic name, and proposed *Streptomyces*, a word derived from the first two names given to the actinomycetes as a whole (*Strepto-thrix* and *Actino-myces*). Since the Botanical Code recommended that family names be derived from generic names, the new family name *Streptomycetaceae* was proposed for the spore-forming actinomycetes.

Streptomycetaceae included actinomycetes with branched, slender substrate mycelium, nonseptate or rarely septate, forming spores on aerial hyphae, and not fragmenting into oidia. Two genera were included in this family: *Streptomyces* and *Micromonospora*. Spores are apparently endogenous in origin, formed by a segregation of protoplasm within the hypha into a series of round, oval, or cylindric bodies. Chains of spores are often spirally coiled. Sporophores may be simple or branched.

Waksman and Henrici selected as the type species of this newly named genus, *Streptomyces albus* (Rossi-Doria emend Krainsky) comb. nov. This species was first described as *Streptothrix alba* Rossi-Doria and later known as *Actinomyces albus* Krainsky. This is one of the commonest and best known species of the genus, and though it may now be recognized as a group and be subdivided into several species, it was considered, for the time being, as definite a species as any others. It produces colorless vegetative growth, with white aerial mycelium, and forms ovoidal spores in coiled chains on lateral branches of the aerial hyphae. It is proteolytic, liquefying gelatin and peptonizing milk with the production of an alkaline reaction in the latter. It does not produce any soluble pigment either on organic or synthetic media, and forms a characteristic earthy or musty odor, as described in detail later.

The generic name *Micromonospora* Ørskov was applied to those forms which produce single spores on lateral branches. Although it was recognized that Tsiklinsky had previously applied the name *Thermoactinomyces* to similar cultures, her description of the genus, in which she also included thermophilic species with catenulate spores, was based on temperature relations rather than on morphology.

On the basis of these and other considerations, Waksman and Henrici proposed a system of nomenclature and classification of the Actinomycetales which has been used as

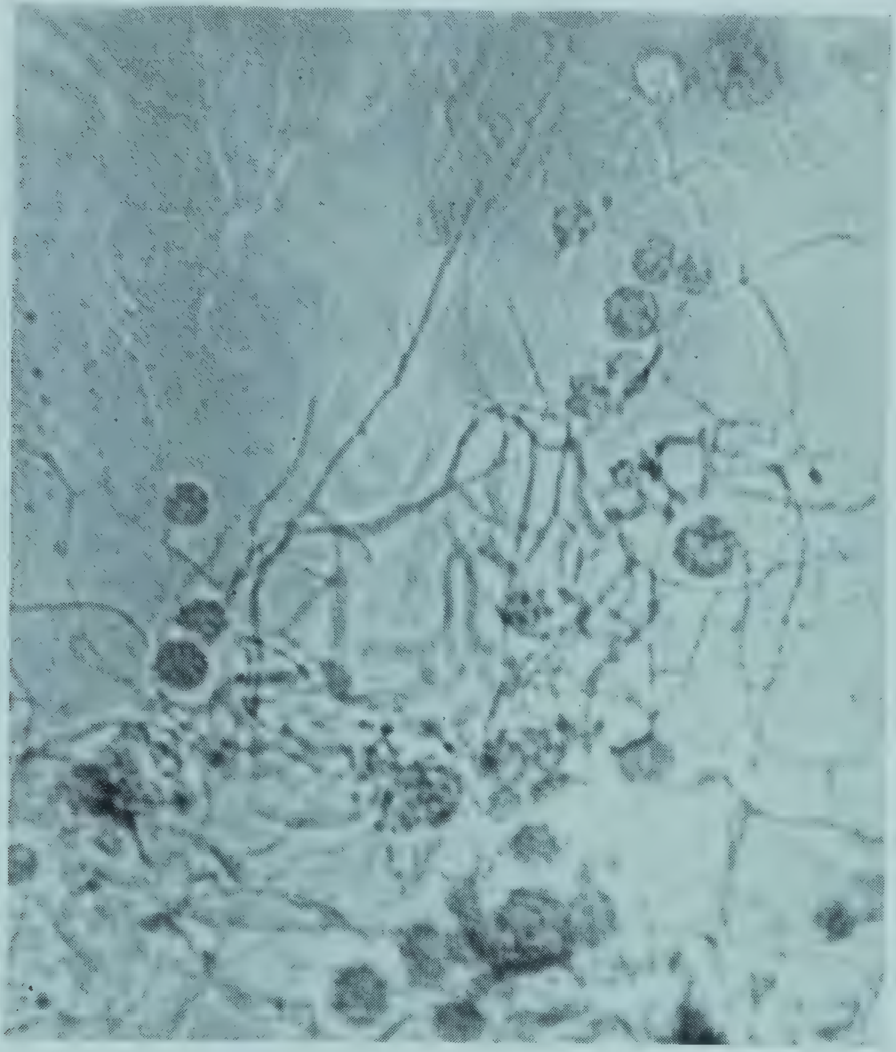


FIGURE 31. The genus *Streptosporangium*.

the basis for the two latest editions of *Bergey's Manual*, and which is becoming generally accepted.

Couch (1950-1955) suggested, on the basis of formation of sporangiospores, the creation of a new family *Actinoplanaceae* with two new genera, *Actinoplanes* and *Streptosporangium*.

Waksman and Corke (1953) proposed to reintroduce the generic name *Thermoactinomyces*, with type species *T. vulgaris* Tsiklinsky, to include the thermophilic forms. The organisms included in this genus are similar in some respects (production of single conidia) to *Micromonospora*, and in others (production of aerial mycelium) to *Streptomyces*. Three species were included in this genus: *T. vulgaris*, *T. monosporus*, and *T. thalpophilus*.

H. and M. Lechevalier proposed (1957) the creation of a genus *Waksmania*, with a type species *W. rosea*, on the basis of formation of double spores. They suggested classification of the *Streptomycetaceae* as follows:

I. Aerial mycelium formed:
  1. Spores formed singly (not in chains). *Thermoactinomyces*
  2. Spores formed in pairs. *Waksmania*
  3. Spores formed in chains. *Streptomyces*

II. Aerial mycelium not formed.
  1. Spores formed singly (not in chains). *Micromonospora*

By a most peculiar coincidence, virtually simultaneously, a similar organism was described and published in Japan, by Nonomura and Ohara (1957), under the name *Microbispora*, with the type species *M. rosea*. These investigators proposed division of the family *Streptomycetaceae* as follows:

- I. Spores formed in chains from aerial hyphae.................*Streptomyces*
- II. Spores formed in pairs on aerial hyphae................*Microbispora*
- III. Spores formed singly on sporophores.
  - 1. Mesophilic.......*Micromonospora*
  - 2. Thermophilic...*Thermoactinomyces*
- IV. Sclerotic granules produced (conidial forms unknown)............*Chainia*

According to Henssen (1957), the majority of thermophilic actinomycetes thrive better under anaerobic than under aerobic conditions; they may, therefore, be designated as facultative aerobes. The preference for anaerobic conditions varies with the species, especially in the case of freshly isolated cultures. When originally isolated under exclusion of oxygen, cultures grow well later also in presence of oxygen, but only at optimum temperature. Production of aerial mycelium is closely bound to optimum temperature. For example, *Thermopolyspora bispora* grows and produces aerial mycelium at 50 to 60°C under aerobic and anaerobic conditions; below that temperature, growth takes place only under anaerobic conditions; at 70°C, growth is good but aerial mycelium and spores are no longer produced.

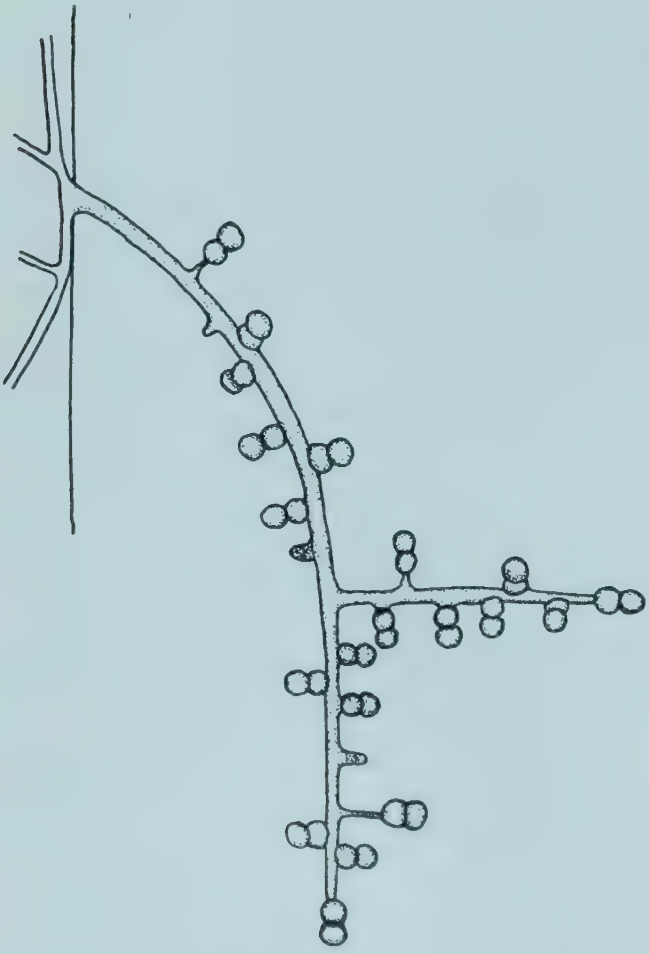


FIGURE 32. *Waksmania rosea* (*Microbispora rosea*), showing the formation of pairs of spores either directly on the spore-bearing hyphae or on short side branches (Reproduced from: Lechevalier, M. P. and Lechevalier, H. J. Gen. Microbiol. 17: 104, 1957).

Henssen proposed division of the family *Streptomycetaceae* into the following genera, taking into consideration the thermophilic forms:

- A. Substrate mycelium nonseptate, spores produced on aerial or substrate mycelium.
  - I. Spores produced on substrate mycelium, mesophilic forms *Micromonospora* Ørskov
  - II. Spores produced on aerial mycelium.
    - 1. Aerial hyphae develop only as side or terminal branches of the substrate mycelium.
      - a. Aerial hyphae are formed as long chains of spores, mesophilic or thermophilic forms *Streptomyces* Waksman and Henrici
      - b. Spores and sporophores produced on the unbranched aerial hyphae.
        - a¹. Spores single, thermophiles *Thermonospora* Henssen
        - b¹. Spores in short chains *Thermopolyspora* Henssen
    - 2. Aerial hyphae develop as side, terminal, and arched-shaped branches of the substrate hyphae growing out of the agar, thermophilic forms *Thermoactinomyces* Tsiklinsky
- B. Substrate mycelium septate, spores formed from the aerial and from the substrate mycelium, thermophilic *Pseudonocardia* Henssen

New genera, as well as numerous species, have thus been added at an ever-increasing rate. Some of these must receive careful consideration, since they are based upon fundamental morphological concepts. Others should be modified, at least for the present, because the differences observed are largely quantitative rather than qualitative. The creation of new species on the basis of their economic importance tends to create confusion in the proper identification of the organisms.

On the basis of these and other modifications of the classification system of Waksman and Henrici, the following classification of the actinomycetes may be presented.

ORDER ACTINOMYCETALES

Organisms forming filamentous cells with a definite tendency to branch. Hyphae do not exceed 1.5 μ and are mostly about 1 μ or less in diameter. Usually producing a characteristic branching mycelium. Multiplication by means of special spores, oidiospores, conidia, or sporangiospores, or combinations of these spores. Special spores are formed by fragmentation of the plasma within straight or spiral-shaped spore-bearing hyphae; the oidiospores are formed by segmentation, or by transverse division of hyphae, similar to the formation of oidia among the true fungi. Conidia produced singly, at the end of simple or branching conidiophores; these spores may be borne singly, in pairs, or in chains. Sporangiospores are borne in spherical or variously shaped sporangia. These organisms grow readily on artificial media and form well-developed colonies. The surface of the colony may become covered with a special aerial mycelium. Some of the organisms are colorless or white, whereas others form a variety of pigments. They are either saprophytic or parasitic. In relation to temperature, most are mesophilic while some are thermophilic. Certain forms are capable of growing at low oxygen tension.

- A. Mycelium rudimentary or absent, no spores formed.
  - Family I. *Mycobacteriaceae* Chester
  - I. Acid-fast organisms
    - *Mycobacterium* Lehmann and Neumann
- B. True mycelium produced, spores formed, but not in sporangia.
  - I. Vegetative mycelium fragmenting into bacillary or coccoid elements.
    - Family II. *Actinomycetaceae* Buchanan
    - 1. Anaerobic or microaerophilic, parasitic, nonacid-fast.
      - 1. *Actinomyces* Harz
    - 2. Aerobic, partially acid-fast or non-acid fast.
      - 2. *Nocardia* Trevisan
  - II. Vegetative mycelium nonseptate, not fragmenting into bacillary or coccoid elements.
    - Family III. *Streptomycetaceae* Waksman and Henrici
    - 1. Aerial mycelium not produced.
      - a. Spores formed singly on short sporophores.
        - a^1. Mesophilic forms.
          - 3. *Micromonospora* Ørskov
        - b^1. Thermophilic forms.
          - 4. *Thermomonospora* Henssen
    - 2. Aerial mycelium produced.
      - a. Spores formed in chains.
        - 5. *Streptomyces* Waksman and Henrici
      - b. Spores formed singly.
        - 6. *Thermoactinomyces* Tsiklinsky
      - c. Spores formed in pairs or in chains.
        - a^1. Mesophilic forms, in pairs.
          - 7. *Waksmania* Lechevalier and Lechevalier
          - (*Microbispora* Nonomura and Ohara)
        - b^1. Thermophilic forms, in pairs or in chains.
          - 8. *Thermopolyspora* Henssen
- C. True mycelium produced as in B above, spores formed in sporangia.
  - Family IV. *Actinoplanaceae.*
  - I. Aerial mycelium usually not formed, coiled conidiophores lacking, sporangiospores motile.
    - 9. *Actinoplanes* Couch
  - II. Aerial mycelium abundant, coiled conidiophores as well as sporangia formed in some species, sporangiospores nonmotile.
    - 10. *Streptosporangium* Couch

The true actinomycetes are thus shown to comprise 10 genera.

Other Developments

The above system has been variously modified and new systems have been proposed. Some of the modifications, especially the various efforts to subdivide the genera into species, were justified. Others were not, especially when new genera were added with insufficient justification.

Gulliermond and Mangenot (1946) suggested division of the *Actinomycetales* into two groups: (a) acid-resistant forms made up of two genera, *Actinomyces* and *Mycobacterium;* (b) nonacid-resistant forms with two genera, *Corynebacterium* and *Pfeifferella.*

Bisset and Moore suggested the creation of two separate orders, Streptomycetales and Actinomycetales, in order to distinguish, by the type of branching, the aerobic sporulating genus *Streptomyces* from the parasitic anaerobic genus *Actinomyces*. The second order was subdivided, on the basis of type and arrangement of the component cells, into two families: 1. *Actinomycetaceae*, including the anaerobic *Actinomyces*, and a new genus, *Jensenia*, comprising the "soil diphtheroids"; 2. *Mycobacteriaceae*, including the genera *Mycobacterium*, *Corynebacterium*, and *Nocardia*.

Thirumalachar (1955) proposed creation of a new genus *Chainia*, with a type species, *C. antibiotica*, on the basis of formation of spherical sclerotic granules, in large aggregate masses, from the mycelium. This phenomenon is frequently observed among *Streptomyces* species, and hardly warrants creation of a separate genus. Gattani (1957), for example, reported the formation of sclerotic granules by various strains of *S. griseus* when grown on certain media. No aerial mycelium is produced under such conditions. A hypha thickens; other hyphae surround it, resulting in brownish green masses, eventually becoming black. Some of the granules coalesce in pairs or in threes, giving rise to a larger granule up to 75 μ in diameter.

Baldacci recently proposed (1958) that all the verticil-forming organisms be united into one genus under the name *Streptoverticillium*. The idea of separating all the forms producing primary or secondary verticils into a separate group was suggested by the writer 40 years ago, and was discarded as hardly advisable. More recently Pridham *et al.* (1957) created four sections in the genus *Streptomyces*, under the names *Monoverticillus* and *Biverticillus*, each comprising either straight or spiral-shaped sporophores. Other investigators attempted to bring these forms together into a distinct genus but were dissuaded by the writer. The fact that the composition of the medium greatly influences this manner of sporulation was among the many reasons advanced against the recognition of a separate genus.

Evidence is gradually accumulating to justify, substantiate, or even enlarge upon the previously established systems of genetic and specific classification. Such evidence is based upon the following four criteria: (a) cell morphology, (b) cell genetics, (c) cell composition, and (d) biochemical activities of the organisms.

Pridham and Gottlieb (1948) suggested division of the actinomycetes on the basis of their carbon utilization. All the streptomycetes tested were able to utilize D-glucose, D-mannose, starch, dextrin, and glycerol, but not erythritol, phenol, the cresols, Na-formate, Na-oxalate, and Na-tartrate. The conclusion was reached that L-rhamnose, raffinose, L-xylose, D-fructose, L-arabinose, and D-mannitol are best for characterizing *Streptomyces* species, as brought out in Chapter 2. This property was utilized by Kurosawa, Gordon and Smith, Zähner and Ettlinger, and others. In spite of the valuable information thus obtained, however, the conclusion may be reached that in no case can the utilization of carbon sources serve alone as a major criterion for characterizing actinomycetes. The information is only supplementary in nature.

Gordon and Mihm divided a total of 676 cultures received under the generic names *Streptomyces*, *Nocardia*, and *Mycobacterium* into groups on the basis of sporulation, as

TABLE 10

Distribution of colonial characters of strains initially designated as Streptomyces, Nocardia, *or* Mycobacterium (Gordon and Mihm)

Original designation (number of strains)	Group 1			Group 2	
	Filamentous colonies; vegetative hyphae projecting extensively, often interlacing, firm, rarely fragmenting			Colonies with halo or outcroppings of filaments and filamentous colonies; filaments rudimentary to rhizoid, fragile, often fragmenting; no aerial hyphae	
	Sporulating aerial hyphae, %	Nonsporulating aerial hyphae, %	No aerial hyphae, %	No colonies with smooth margins, %	Colonies with smooth margins, %
Streptomyces (219).........	83	9	8	0	0
Nocardia (214)............	24	47	10	2	16
Mycobacterium (243).......	0	0	0	5	89

shown in Table 10. These results are of great significance in interpreting the identity of a particular culture received under a given name.

Schneidau and Shaffer carried out comparative cultural studies with 51 cultures received under the name of *Nocardia*, 10 cultures designated as *Mycobacterium*, and four as *Streptomyces*. Characteristics that appeared to be stable and of value for group or specific identification were: (a) color of the stroma under uniform cultural conditions, (b) temperature tolerance, (c) hydrolysis of starch, (d) liquefaction of gelatin and/or hydrolysis of casein. Such characteristics as paraffin utilization, catalase activity, or indol production appeared to be of little value for identification inasmuch as virtually all strains studied behaved similarly, irrespective of morphologic or other physiologic differences.

Inability to produce a submerged mycelium appeared a useful criterion to distinguish strains of *Mycobacterium* from markedly fragmenting *Nocardia* with which they might otherwise be confused. Production of aerial mycelium and diffusible pigment, degree of fragmentation and acid-fastness, hemolytic and urease activity have only limited value as taxonomic criteria within the genus *Nocardia* but may be helpful in distinguishing among the genera *Mycobacterium, Nocardia*, and *Streptomyces*. *N. intracellularis* did not behave culturally like a *Nocardia* but appeared more closely related to the genus *Mycobacterium* (Table 11).

TABLE 11

Effect of composition of medium on aerial mycelium formation by strains of Nocardia *and* Mycobacterium (Schneidau and Shaffer)

Strain	Aerial mycelium on			
	Synthetic agar	Potato glucose agar	Sabouraud's agar	Glycerol agar
N. asteroides...........	+	−	−	+
N. asteroides...........	+	+	+	+
N. asteroides...........	−	−	+	+
N. blackwellii..........	−	−	+	−
N. cuniculi.............	−	−	−	+
N. minima...............	+	+	+	+
N. paraffinae...........	+	−	−	−
N. polychromogenes.....	+	+	+	+
N. brasiliensis.........	+	+	−	+
N. brasiliensis.........	+	+	+	+
N. corallina............	−	−	−	−
N. erythropolis.........	−	−	−	−
N. globerula............	−	−	−	−
N. opaca................	−	−	−	−
N. leishmanii...........	−	+	−	−
N. madurae..............	−	+	−	−
N. madurae..............	+	+	+	+
N. pelletieri...........	−	−	−	−
N. rangoonensis.........	+	+	+	+
N. intracellularis......	−	−	−	−
M. butyricum............	−	−	−	−
M. leprae...............	−	−	−	−

TABLE 12

Cultural characteristics of strains of Nocardia, Streptomyces, *and* Mycobacterium (Schneidau and Shaffer)

Strain	Gross culture				Slide culture		Physiologic and biochemical properties							
	Color of substrate growth		Starch agar		Starch agar Surface growth									
	Yellow, orange, red	White, cream, tan, buff, or brown	Diffusible pigment	Aerial mycelium	Branched mycelium	Fragmentation	Acid-fastness	Growth at 46° C	Paraffin utilization	Diastasis	Gelatin liquefaction	Casein hydrolysis	Urease	Hemolysis
N. asteroides Henrici A10A	+	−	+	+	+	+	+	+	+	−	−	−	−	+
N. asteroides ATCC 9504	+	−	+	+	+	+	+	+	+	−	−	−	+	−
N. asteroides ATCC 9970	+	−	−	−	+	+	+	+	+	−	−	−	+	−
N. asteroides ATCC 3308	+	−	−	+	+	+	−	+	+	−	−	−	+	+
N. asteroides ATCC 9969	−	+	+	+	+	+	+	+	+	−	−	−	+	−
N. asteroides NRRL B970	+	−	+	+	+	+	+	+	+	−	−	−	+	+
N. blackwellii ATCC 6846	+	−	−	−	+	+	+	+	+	−	−	−	+	−
N. caprae 373	+	−	+	−	+	±	+	+	−	−	−	−	+	−
N. cuniculi ATCC 6864	+	−	−	+	+	+	+	+	+	−	−	−	+	−
N. sylvodorifera ATCC 7372	+	−	−	+	+	+	−	+	+	−	−	−	+	−
N. minima ATCC 8674	+	−	+	+	+	+	+	−	+	−	−	−	+	+
N. paraffinae 3410	+	−	+	+	+	+	+	−	+	−	−	−	+	−
N. polychromogenes ATCC 3409	+	−	+	+	+	+	−	−	+	−	−	−	+	−
N. brasiliensis Ochoa 409	+	−	+	−	+	+	+	−	+	−	+	+	+	+
N. brasiliensis 2178	+	−	−	−	+	+	+	−	+	−	+	+	+	+
N. convoluta ATCC 4275	+	−	−	−	±	+	+	−	+	−	−	−	+	−
N. corallina ATCC 999	+	−	−	−	±	+	+	−	+	−	−	−	+	+
N. erythropolis ATCC 4277	+	−	−	−	±	+	+	−	+	−	−	−	+	+
N. globerula ATCC 9356	+	−	−	−	±	+	−	−	+	−	−	−	+	+
N. opaca ATCC 4276	+	−	−	−	±	+	+	+	+	−	−	−	−	+
N. rubra NRRL B685	+	−	−	−	±	+	−	+	+	−	−	−	−	+
N. caviae ATCC 6848	−	+	−	+	+	−	−	+	+	+	+	+	−	+
N. gardneri ATCC 9604	−	+	+	+	+	−	−	−	−	+	+	+	−	+
N. leishmanii ATCC 6855	−	+	−	+	+	−	−	−	+	+	+	+	±	+
N. madurae Ochoa 415	−	+	−	−	+	−	−	+	+	−	+	+	−	+
N. madurae ATCC 6245	−	+	+	+	+	−	−	+	+	+	+	+	−	+
N. pelletieri Lacaz 293	+	−	−	−	+	−	−	+	−	−	+	+	−	−
N. pelletieri 47293	+	−	−	−	+	−	−	+	−	−	+	+	−	+
N. rangoonensis ATCC 6860	−	+	+	+	+	−	−	+	+	−	+	+	+	+
N. intracellularis 330	−	+	−	−	−	+	+	−	+	−	−	−	−	−
S. aureus ATCC 3309	+	−	−	+	+	−	−	+		+	+	+	+	+
S. griseus ATCC 3326A	−	+	−	+	+	−	−	−		+	+	+	−	+
S. ruber ATCC 3348	−	+	+	+	+	−	−	+		+	+	+	−	+
S. venezuelae ATCC 10595	−	+	+	+	+	−	−	+		+	+	+	−	+
M. butyricum 264	−	+	−	−	−	+	+	+	+	−	−	−	+	−
M. leprae Duval 104	+	−	−	−	−	+	+	+	+	−	−	−	+	−
M. phlei W23	+	−	−	−	−	+	+	+	+	−	−	−	+	−
M. smegmatis 270	+	−	−	−	±	+	+	+	+	−	−	−	+	−
M. stercoris 262	−	+	−	−	−	+	+	+	+	−	−	−	+	−

The strains of *Nocardia* studied were separated, on the basis of the cultural data presented, into "true" *Nocardia* and "streptomyces-like" *Nocardia*. The former have been further tentatively divided into the following groups: (a) *N. asteroides* group (all strains of *N. asteroides* plus *N. blackwellii*, *N. cuniculi*, *N. caprae*, *N. sylvodorifera*, *N. polychromogenes*, *N. minima*, and *N. paraffinae*); (b) *N. corallina* (*N. globerula*, *N. erythropolis*, and *N. convoluta*); and (c) *N. opaca* group (*N. opaca* and *N. rubra*) (Table 12).

Various other criteria have been suggested as aids in the classification of the actinomycetes, notably members of the genus *Streptomyces*. It is sufficient to mention phage susceptibility (Stocker) and formation of antibiotics (Kurosawa, Kuroya, *et al.*, 1950). According to Krassilnikov (1957), antibiotics may be considered as necessary in the struggle for life between rival organisms. They manifest their activity toward competing organisms only, but never against cultures of the same species. He believed that strains of one species produce antibiotics inhibiting the growth of all the strains of rival species. This antagonism may be unilateral or bilaterial. He suggested that the specificity of antibiotics produced by various species be used in taxonomy for the differentiation of these species. He reported success in demonstrating, by means of cross antagonism experiments, the heterogeneity of many species which heretofore were considered as homogeneous.

Waksman and Lechevalier found that, on the basis of sensitivity to isoniazide, the phylogenic position of the various Actinomycetales was indicated as follows: The pathogenic forms of the genus *Mycobacterium* are susceptible to less than 1 mg of isoniazide per milliliter, the avian form being least susceptible; the saprophytic forms were less susceptible, some being resistant to 100 and even 1000 mg per milliliter, most of them being susceptible to less than 5 and 10 mg per milliliter. The micromonosporas were more resistant to isoniazid than the mycobacteria (10 to 1000 mg per milliliter). The nocardias were also moderately more resistant, most of them requiring 80 to 300 mg of isoniazide per milliliter for growth inhibition; some were more susceptible (10 mg per milliliter), and others were resistant (more than 1000 mg per milliliter). The streptomycetes were most resistant, requiring as a rule more than 1000 mg of isoniazide per milliliter for inhibition. The various genera of the Actinomycetales could thus be listed in the following order of increasing resistance to isoniazide: pathogenic mycobacteria → saprophytic mycobacteria → *Micromonospora* → *Nocardia* → *Streptomyces*.

Among the new approaches to the tax-

TABLE 13

Classification of the actinomycetes based on cell wall composition (Commins and Harris)

Family	Genus	Cell wall components	
		Sugars	Amino acids
Mycobacteriaceae	*Nocardia* *Mycobacterium* *Corynebacterium*	Arabinose, galactose	Alanine, glutamic acid, DL-diaminopimelic acid
Actinomycetaceae	*Actinomyces*	Galactose	Alanine, glutamic acid, lysine
Streptomycetaceae	*Streptomyces* *Micromonospora* (*Propionibacterium*)*	No characteristic sugar	Alanine, glutamic acid, glycine, LL-diaminopimelic acid†

* Position rather doubtful.

† In *Micromonospora* DL-diaminopimelic acid is present in addition.

onomy of actinomycetes, that based upon the chemical composition of the cell wall deserves particular consideration (see Chapter 9). The results tended to emphasize the close relationship of the actinomycetes to the bacteria and not to the Eumycetes or true fungi. Cummins and Harris (1958) found that the cell walls of the actinomycetes were made up of sugars, amino sugars, and a few amino acids (usually three or four), the general pattern of these components being identical with that of the gram-positive bacteria. The mycelial walls of the fungi are composed entirely of carbohydrate. They proposed a system of classification of the actinomycetes based upon the chemical composition of their cell walls (Table 13). They even went as far as to suggest that the order Actinomycetales be abolished altogether and the families of actinomycetes be included with the Eubacteriales.

Recognition of Certain Groups Among the Actinomycetes

In order to complete the historical background of classification of actinomycetes, one further aspect must be mentioned, and that is the recognition by many of the earlier investigators that certain individual species of actinomycetes may just as well be given a group characteristic. Sanfelice suggested in 1904 that the actinomycetes be divided into three groups as follows:

1. *Streptothrix alba.* Colonies opaque to white, covered with calcareous powder, and adhering fast to the medium. On potatoes, growth is rapid, white, with a lime-like surface; pigment remains unchanged, or may become gray; occasionally the color may change to black or straw-yellow. Sanfelice emphasized that "the superficial observer could create out of a dark culture a new species, without recognizing the original nature of *S. alba.*"
2. *Streptothrix flava,* frequently obtained from the dust, shows much variation in pigmentation. Growth lichenoid, intensely yellow. On potatoes, growth is also lichenoid, but color less intense. Color may gradually change on continued transfer, becoming either lighter or deeper orange-red. Apparently, this group comprised forms that are now largely considered as *Nocardia* species.
3. *Streptothrix violacea.* Opaque, lichenoid growth, brownish in color, occasionally turning black. On potato, growth is of a bluish amethyst color.

Foulerton, Chalmers and Christopherson, Langeron, and Brumpt also suggested division of the pathogenic species into several sections or groups. These were designated as Breviores (*A. bovis, A. israeli*), Minores (*A. asteroides*), and Majores (*A. albus, A. chromogenes*). These sections correspond to the first three genera in the Waksman and Henrici classification, namely, *Actinomyces*, *Nocardia*, and *Streptomyces*.

Further systems of classification of the various genera, notably *Streptomyces*, into groups, sections, and series, each of which comprises a number of species, will be discussed in detail in Vol. II, Chapters 20 and 21.

CHAPTER 5

Morphology, Cytology, and Life Cycles

General Morphological Properties of Actinomycetes

As has been pointed out, all the evidence recently submitted concerning the structure and functions of actinomycetes definitely establishes the fact that these organisms are to be classified with the bacteria and not with the fungi. Lehmann and Neumann (1896) were among the first to draw attention to the close morphological relationship between the diphtheria and the tubercle bacillus, on the one hand, and the actinomycetes on the other. Later (1920), they emphasized that the distinction between mycobacteria and actinomycetes is not very sharp, inasmuch as some mycobacteria show only slight resistance to decolorization by mineral acids, and some of the actinomycetes possess relatively well-developed acid-fastness. As pointed out previously, Cummins and Harris suggested, on the basis of recent chemical evidence, that the order Actinomycetales be abolished altogether and that the families of the actinomycetes be included in the Eubacteriales.

Actinomycetes, like the true bacteria, are procaryotes. Their growth (protothallus) is made up of branching filaments, producing a mycelium. This may be of two types, one prostrate, forming a vegetative growth, sometimes referred to as substrate mycelium; the other, erect or aerial mycelium. The spore-bearing hyphae of the aerial mycelium usually have a somewhat greater diameter than the hyphae of the substrate mycelium. These two types of mycelium, or mycelial stages, are not only structurally different but possess different growth requirements. The secondary or aerial mycelium is considered by most investigators to originate asexually from the primary or substrate mycelium; some consider it as a sexual stage.

Actinomycetes produce two types of spores: (a) true conidia, and (b) arthrospores or chlamydospores. The earlier investigators, notably Lachner-Sandoval, recognized "fragmentation" spores appearing as spherical to cylindrical segments of old hyphae, produced by the contraction of the protoplasm; and "segmentation" spores produced by the septation of the tips of the aerial filaments, usually formed in lateral branches of the aerial hyphae but also extending to the main filaments in substrate growths. According to Neukirch, the "segmentation" spores are produced not by a process of septation of the aerial mycelium, but by the successive contractions of the protoplasm, until approximately isodiametric portions are separated by regularly alternating empty spaces (see also Domec).

On the basis of a study of a number of saprophytic actinomycetes, belonging to the genus now recognized as *Streptomyces*, Drechsler summarized their morphological characteristics as follows:

> 1. The vegetative growth consists of a mycelium composed of profusely branching hyphae, the terminal growing portions of which are densely

filled with protoplasm; toward the center of the thallus, the vacuoles increase in size, and may be associated with the presence of metachromatic granules. The vegetative hyphae of the mycelium are far larger than those of ordinary bacteria of the acid-fast group; the hyphae also lack the uniformity in diameter generally characteristic of the true bacteria.

2. The aerial mycelium produced on suitable media usually occurs as a mat of discrete fructifications. Each of these represents a well-characterized sporogenous apparatus, consisting of a sterile axial filament bearing branches in an open racemose, or dense capitate, arrangement. The primary branches may function directly as sporogenous hyphae, or they may proliferate branches of the second and of higher order; sporogenesis, in the latter case, is confined to the terminal elements, the hyphal portions below points of attachment of branches remaining sterile.

3. Two tendencies in the development of fructifications were recognized: (a) one leading to an erect dendroidal type, in which successively proliferated fertile elements undergo processes of sporogenesis in continuous sequence; (b) the other leading to a prostrate, racemose type, in which sporogenesis is delayed in the older branches until the younger branches have also attained their final extension. The majority of species show these tendencies combined in different ways.

4. The sporogenous hyphae of many species are coiled in peculiar spirals. These exhibit pronounced specific characteristics in the number, diameter, and obliquity of their turns, and especially in the direction of rotation, which may be dextrorse or sinistrorse. This phenomenon was later found not to be constant, however, but to vary with the composition of the medium.

5. Sporogenesis begins at the tips of the fertile branches and proceeds basipetally. In some species the process involves the insertion of septa, which are, in certain cases, relatively very massive, and in others, so thin as to be barely discernible.

6. Granules which possess the staining properties and uniformity of size characteristic of nuclei are readily differentiated in the spores of many species; they generally occur singly, but in the larger spores of a few forms, two are often found occupying diagonally opposite positions. As in the vegetative thallus, metachromatic granules occur in the aerial mycelium, being very rarely found in spores or sporogeneous hyphae but becoming very abundant in degenerate sterile hyphae.

7. Peculiar spherical structures appear regularly in some forms, both in the sterile axial hyphae, where they may contain either a medium septum or a number of peripheral metachromatic granules, and in the sporogenous hyphae, where they are associated with the regularly spaced septa.

8. The spores germinate readily in proper media, producing from one to four germ tubes, the approximate number being more or less characteristic of the species.

Ørskov divided the actinomycetes into three morphological groups:

I. Those that produce an undivided substrate mycelium and an aerial mycelium which breaks up into bodies that possess the quality of spores.

II. Those that produce an initially undivided substrate mycelium. After having reached a certain point, it divides by septa into rod-shaped elements; these continue to multiply with a characteristic "angular" growth; aerial mycelium may or may not be formed; in the former case its elements resemble those of the vegetative mycelium.

III. Those that produce a substrate mycelium resembling that of group I, but devoid of aerial mycelium and producing spores borne singly at the distal end of short mycelial branches.

According to Ørskov the angular growth into which the members of group II pass after the formation of an initial mycelium is similar to the process of cell division in the mycobacteria and corynebacteria. This similarity makes it impossible to draw a sharp line of demarcation between true bacteria and actinomycetes.

Jensen emphasized that the nocardias, or proactinomycetes, as he designated them, represent a heterogeneous collection of types, standing between the mycobacteria and corynebacteria, on the one hand, and the streptomyces, on the other.

The morphological structure of actinomycetes depends largely upon (a) the nature of the organism, (b) the composition of the medium, (c) conditions of growth, especially aeration, and (d) presence of growth-stimulating and growth-inhibiting factors. The thallus consists of a homogeneous cytoplasm which, as it grows older, becomes vacuolated and shows refringent granules probably consisting of volutin (Lieske). Fat granules and occasional vacuoles containing chromotropic granules were observed by Grigorakis (1931) in the thallus of the organisms grown on an agar medium containing glycerol and peptone.

Our knowledge of the morphology of actinomycetes has recently been enlarged greatly by studies of their cytology, especially through the use of the electron microscope. Discoveries of chromatic substance (Schaede, von Plotho, 1940), of lipids (Erikson, 1947) and of the chemical composition of the cells (Romano and Sohler, Cummins and Harris) have further contributed to a better understanding of their structural properties.

Electron microscope studies of the mechanism of spore formation by members of the genus *Streptomyces* tended to confirm the view of Lachner-Sandoval, presented as far back as 1898. According to Vernon (1955),

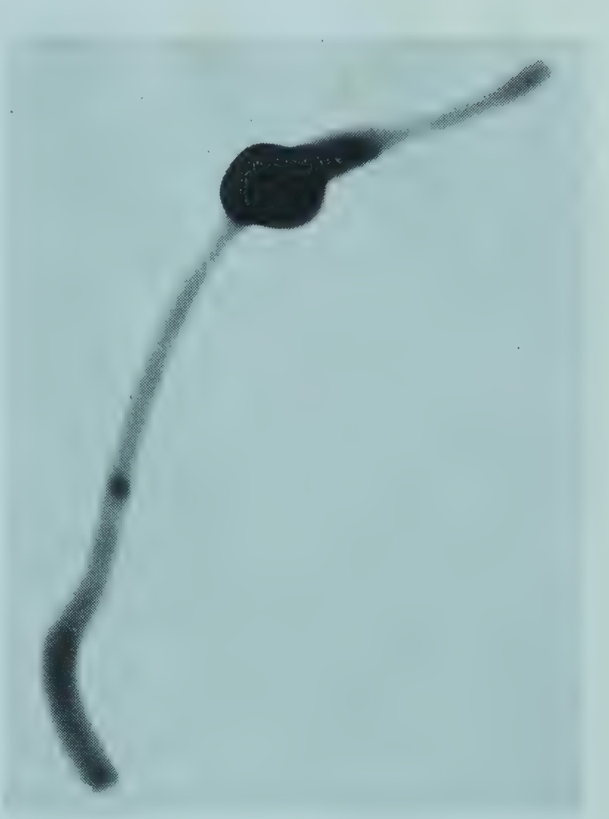


FIGURE 33. Germination of a streptomyces spore, as shown by electron microscopy, ×38,000 (Carvajal, F. Mycologia 38: 589, 1946).

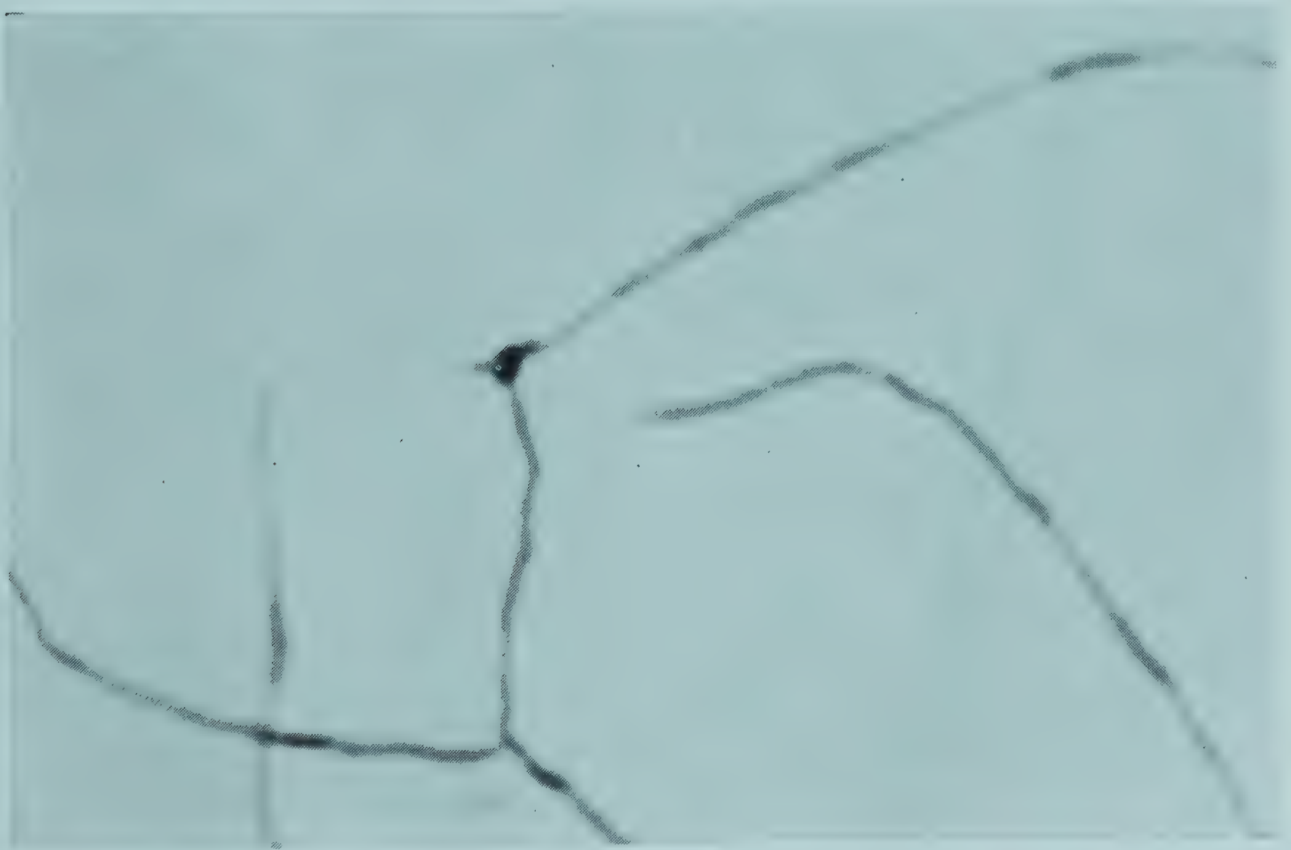


FIGURE 34. *S. griseus:* primary mycelium at 40 hours from submerged culture, showing germinating initial cell. Visual light, × 2000 (Reproduced from: Dickenson, P. B. and Macdonald, K. D. J. Gen. Microbiol. 13: 89, 1955).

spore formation takes place within the hyphal wall. The hyphal contents divide simultaneously into fragments, separated by less dense partitions, having the appearance of septa. The spores remain in chains, held together by a sheath-like hyphal wall, which undergoes change as the spores mature. In some cultures, the sheath persists, the spores being liberated by means of a longitudinal split; the old wall remains as a ribbon-like sheath, with cross markings indicating the position of the spores; in other cultures, the sheath disintegrates to small fragments. The surface structure appearance of the spores varies for the different organisms and is believed by some to be a species characterization (Ettlinger *et al.*, 1958). The formation of spines on the surface of the spores, demonstrated by Flaig *et al.* (1952), Enghusen (1955), Baldacci and Grein, and others, has also been reported by Vernon for *S. flaveolus*, for example.

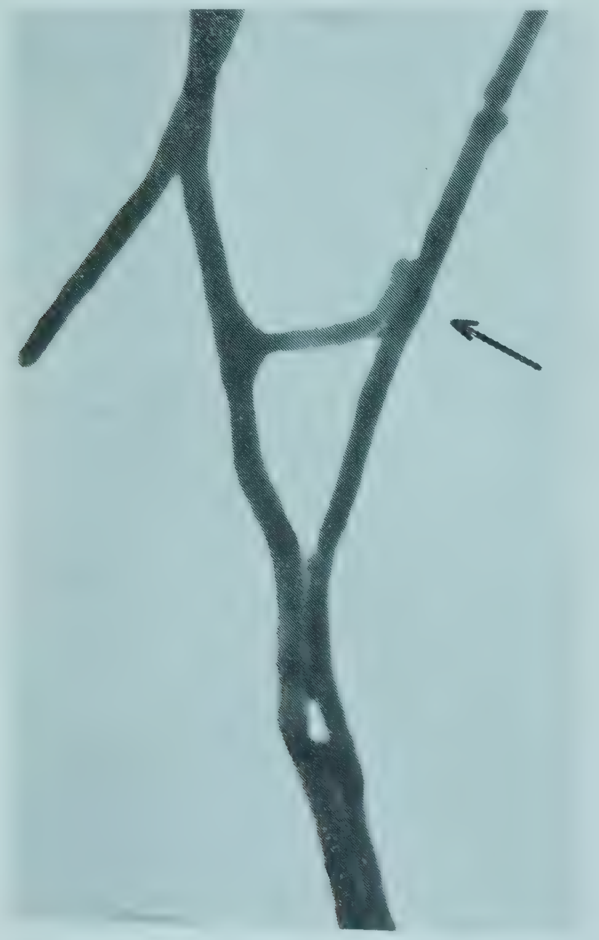


FIGURE 35. *S. griseus:* primary mycelium at 53 hours from submerged culture, showing hyphal fusions. Electron micrograph, × 5000 (Reproduced from: Dickenson, P. B. and Macdonald, K. D. J. Gen. Microbiol. 13: 89, 1955).

Further information on the structure of the mycelium and spores of various actinomycetes is found in the work of Elisei (1944), Carvajal (1946–1947), Scotti and Gocchi, Webley (1955), and many others.

Sexuality among Actinomycetes

The fusion of mycelial threads among actinomycetes has been observed first by Lieske, then by Korber (1929), and others. According to Krassilnikov (1938), this can take place either as the confluence of germinating spores, through the germination tubes, which give rise to a single hypha developing into a mycelium, or as the anastomosis of two hyphal filaments. Krassilnikov suggested that this actually is or resembles the sexual process comparable to that which takes place in many yeasts. He further emphasized the great biological significance of this phenomenon, which possibly explains the variability of the actinomycetes.

According to Klieneberger-Nobel the morphological changes in the growth of a streptomyces on the surface of a medium take place as follows: The spores germinate, giving rise to a substrate or "primary mycelium," which undergoes anastomosis, resulting in the formation of "initial cells," or "fusion cells;" these produce, on germination, aerial or "secondary" mycelium, which sporulates to give rise to spores. This investigator considered untenable the description of spore formation, by Lieske and Orskov, as taking place without any previous segmentation of the protoplasm. During the process of spore formation, the hyphae were believed by Klieneberger-Nobel to be separated by transverse septa into small cells, each of which eventually develops into a spore.

Erickson (1949) suggested that such "ini-

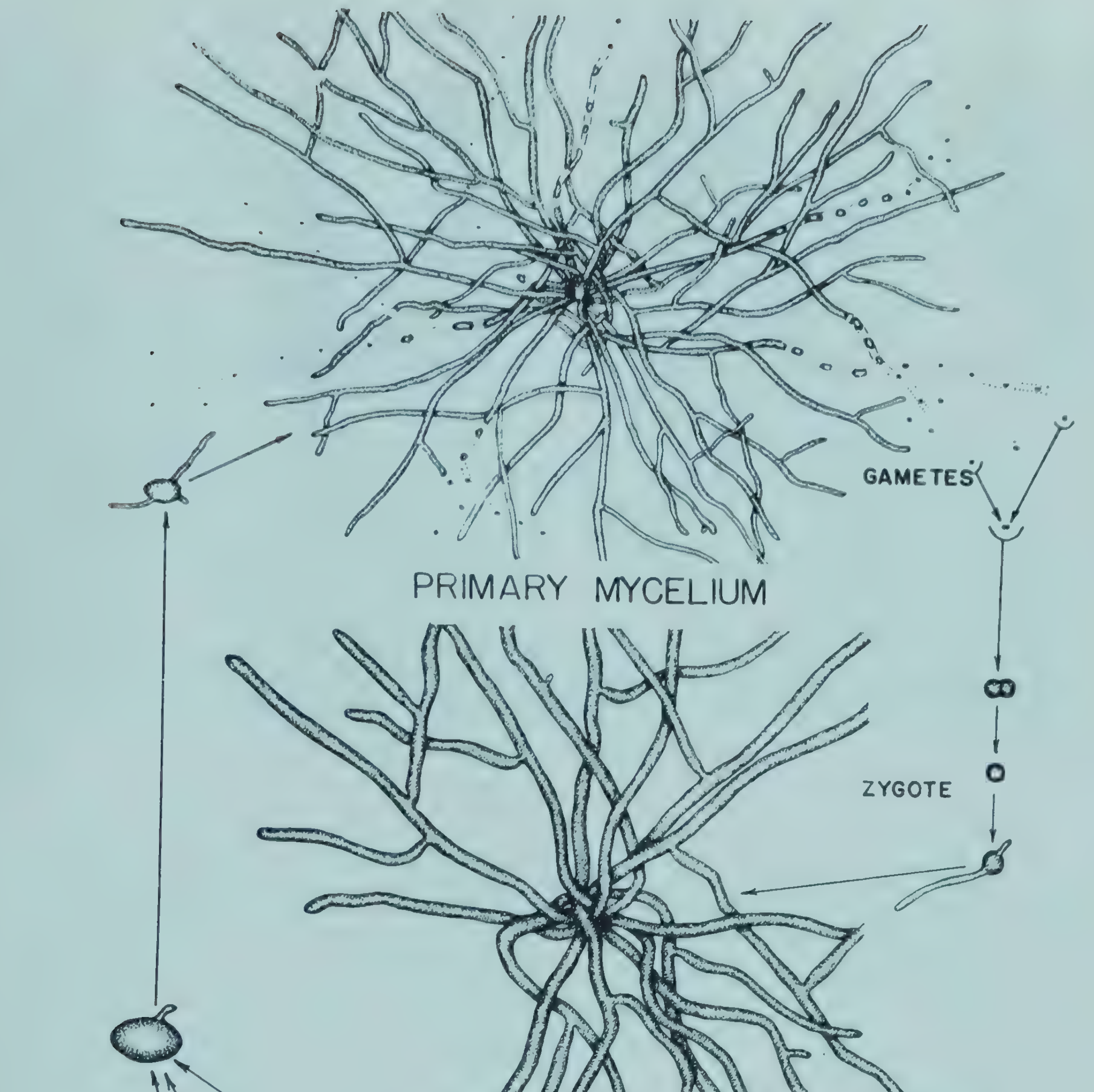

PRIMARY MYCELIUM
GAMETES
ZYGOTE
SECONDARY MYCELIUM
"TETRACYTES"

FIGURE 36. Diagrammatic life cycle of a streptomyces. The primary and secondary mycelia are separated for convenience of illustration though usually the latter originates within the former. Attention is called to the gradual shrinkage of endofragments of the primary mycelium to produce isogametes; the secondary mycelium may be bisporulative (Reproduced from: Roach, A. W. and Silvey, J. K. G. Trans. Am. Microscop. Soc. 77: 36, 1958).

tial cells" are artifacts; she believed that aerial hyphae may arise by budding from any substrate (vegetative) hyphae. Wilkin and Rhodes observed that the nature of the medium influences the morphological forms produced by a *Streptomyces*: on synthetic media, the cycle proposed by Klieneberger-Nobel was confirmed; on complex media, the formation of "initial cells" and "secondary mycelium" was suppressed, however. The "primary mycelium" proliferates on complex media to give "chlamydospores," which, on germination, give rise to typical "primary mycelium." Further information on the life cycles of actinomycetes is given by Wilkin and Rhodes, and Roach and Silvey.

Dickenson and Macdonald made electron microscopic observations on submerged cultures of two species of *Streptomyces*. The evidence obtained tended to confirm the theory of Klieneberger-Nobel, that at an early stage in the life history of the organism concerned, fusion occurs between portions of the same or different hyphae, culminating in the formation of "initial cells."

Further studies on problems of recombination of nuclear material and on the life cycles of actinomycetes in general, are discussed in Chapter 6.

Colony Formation

The growth of an actinomycete on a solid or in a liquid medium results in the formation of a mass of unicellular mycelium usually designated as a "colony." This is not a colony in a true sense, since it is not an accumulation of many cells, but rather a mass of branching filaments which originated from a spore or from a bit of mycelium. The two types of mycelium making up a colony of a streptomyces often show fundamental differences in appearance, composition, and biological activities. The substrate or vegetative mycelium grows into the medium, whereas the aerial mycelium grows on the surface; the well-developed sporulating hyphae and the reproductive spores are produced in the aerial mycelium. Some actinomycetes form only the substrate mycelium, whereas others produce both types. Some aerial mycelium-forming cultures may lose this property, and may thus be distinguished from nocardias only by certain physiological properties, as pointed out elsewhere. Some nocardias, on the other hand, also produce a typical aerial mycelium, as shown by Gordon and Mihm (1958).

According to Henrici (1930), surface colonies produced by various actinomycetes are of two general types: (a) One type is characteristic of those strains that form a highly developed, extensively branching mycelium, notably members of the genus *Streptomyces*. Colonies of this type are very firm, almost cartilaginous in consistency, and adhere to the solid substrate, because the mycelium grows into that substrate; when touched with a wire loop, the colony does not break but separates from the substrate as a unit. In cross section, such colonies usually have a slightly conical form and show marked radial foldings. At first their surface may be glossy or matted, but, if aerial spores are developed, the surface becomes covered with chalk-like powder which, as the colony grows older, may acquire various shades of color. The powdery spores frequently appear in concentric rings. (b) The second type of colony is characteristic of the strains that do not form an extensive mycelium, notably members of the genus *Nocardia*. Their thallus has a tendency to break up into hyphae of variable length, and in certain strains most of the growth may consist of short filaments, resembling in appearance pleomorphic bacterial strains. Colonies of this type are less tenacious than those of the first; they often have a mealy consistency and tend to crumble when touched with a wire loop.

Actinomycete colonies are usually round and smooth, or much folded and lichenoid in appearance. When examined under the mi-

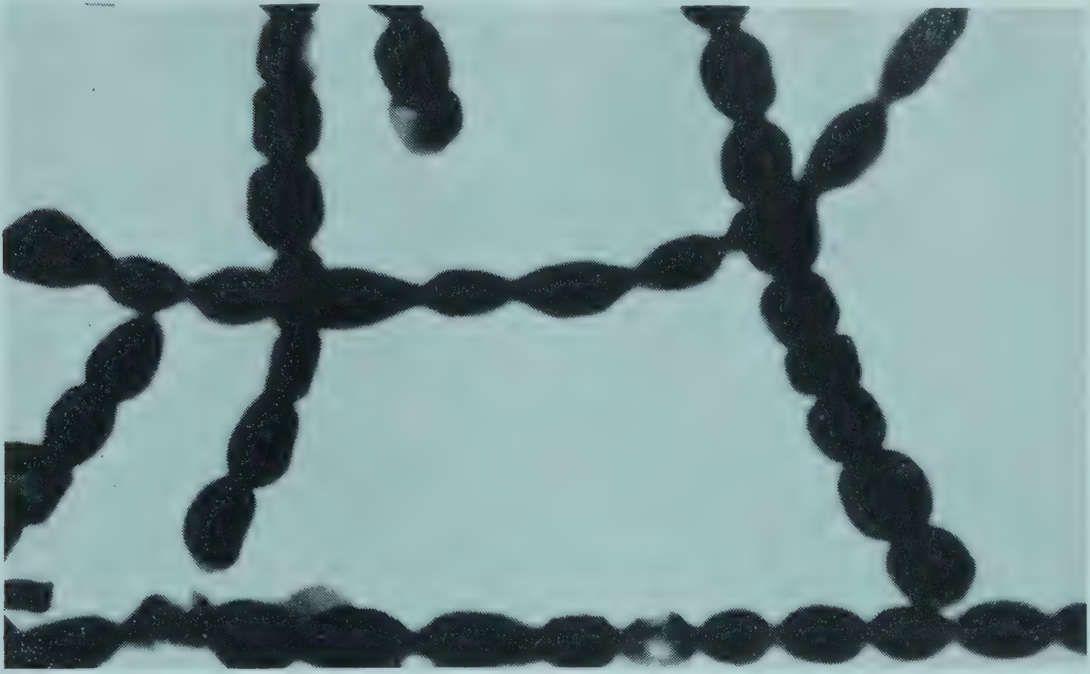


FIGURE 37. Sporulation of *S. griseus* (Reproduced from: Baldacci, E. and Grein, A. Giorn. Microbiol. 1: 34, 1955).

croscope, the edge of the colony shows a characteristic picture of radiating hyphae. When grown in liquid media in a stationary condition, the colonies may be formed individually on the bottom of the container, or they may adhere to the surface of the wall, or they may form a ring of growth or a pellicle on the surface. The colonies may also grow in the form of flakes, but the medium is never made turbid, as in the case of bacterial growth, unless the colonies undergo lysis through the action of enzymes or phages. When grown in a submerged or in a shaken condition, actinomycetes produce small, bead-like masses of growth, some of which may be granular in nature.

According to Jensen, strains of *Nocardia* with more persistent mycelium produce firm agar colonies and a discretely granular growth in liquid media, which remain clear; those with small or rapidly dividing mycelium show a soft growth in solid media and a diffuse, bacterium-like growth in liquid media. Jensen took exception to Lieske's (1921) statement that "every form of turbidity of a liquid medium is to be looked upon as evidence of contamination." He also pointed out that a similar difference exists in cultures of the genus *Actinomyces*, where most strains show a granular growth but some give a cloudy growth in broth (Erikson, 1940; Holm, 1948). The colonies of the bovine strains of this anaerobic genus *Actinomyces* are smooth and soft in consistency and do not adhere to the medium; the mycelium undergoes fragmentation very rapidly, giving no extensive ramification; such strains show occasional turbidity in the medium. In contrast, human strains give no stable variants and produce no turbidity (Erikson).

Various attempts have been made to divide the actinomycetes into groups on the basis of the size of the colonies (Krainsky) or the length of hyphae (Lieske). These properties are not recognized now as of primary significance in classification, since the nature of the organisms and the conditions of growth are of prime importance.

Staining Properties

The mycelium of the actinomycetes can be dried on slides and stained with ordinary aniline dyes. Methyl violet, carbol-fuchsin, and methylene blue can be used. Fresh my-

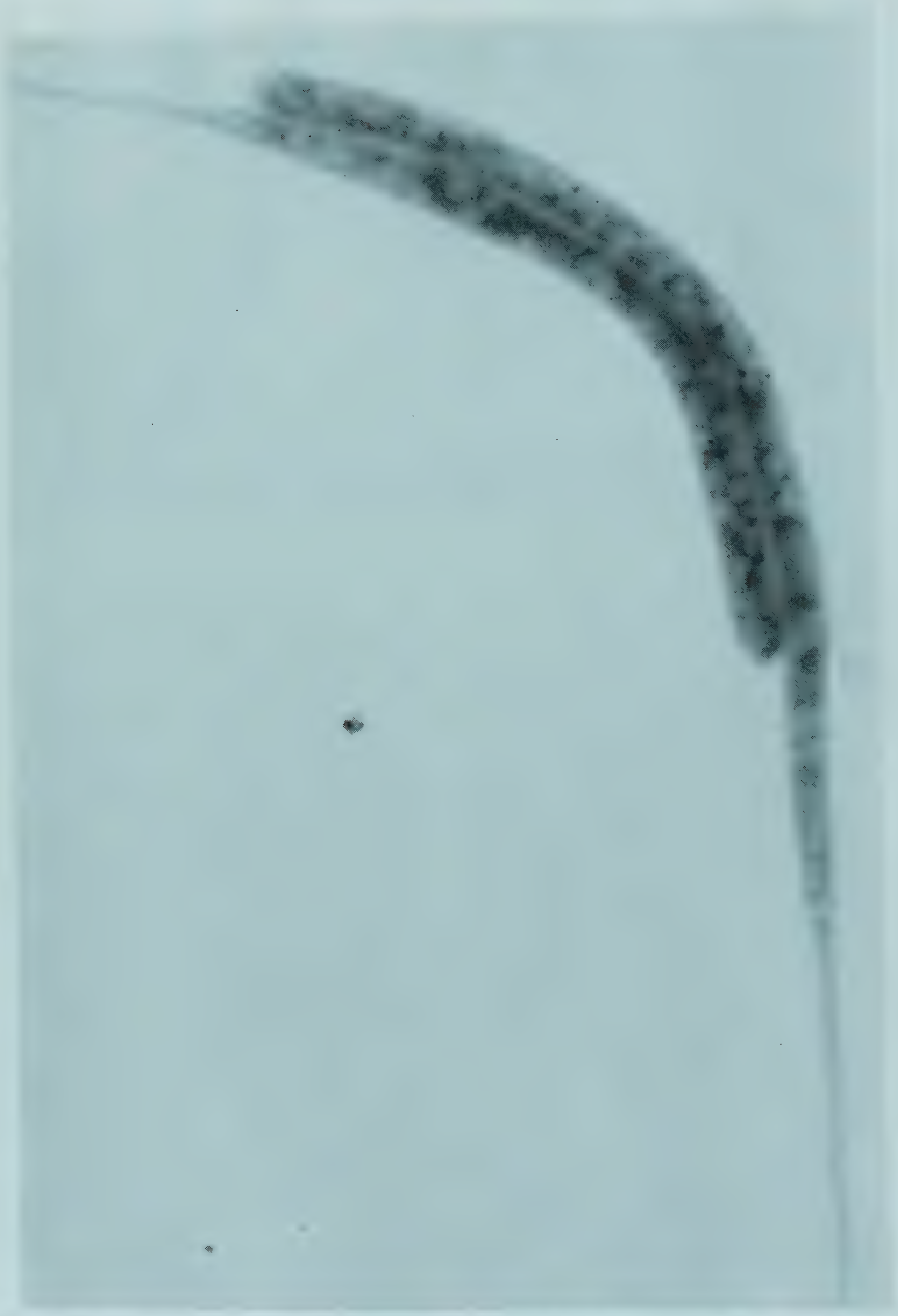


FIGURE 38. *S. griseus:* first stage of initial cell formation from double hyphal contact. From 6-day submerged culture. Electron micrograph, × 10,000 (Reproduced from: Dickenson, P. B. and Macdonald, K. D. J. Gen. Microbiol. **13:** 89, 1955).

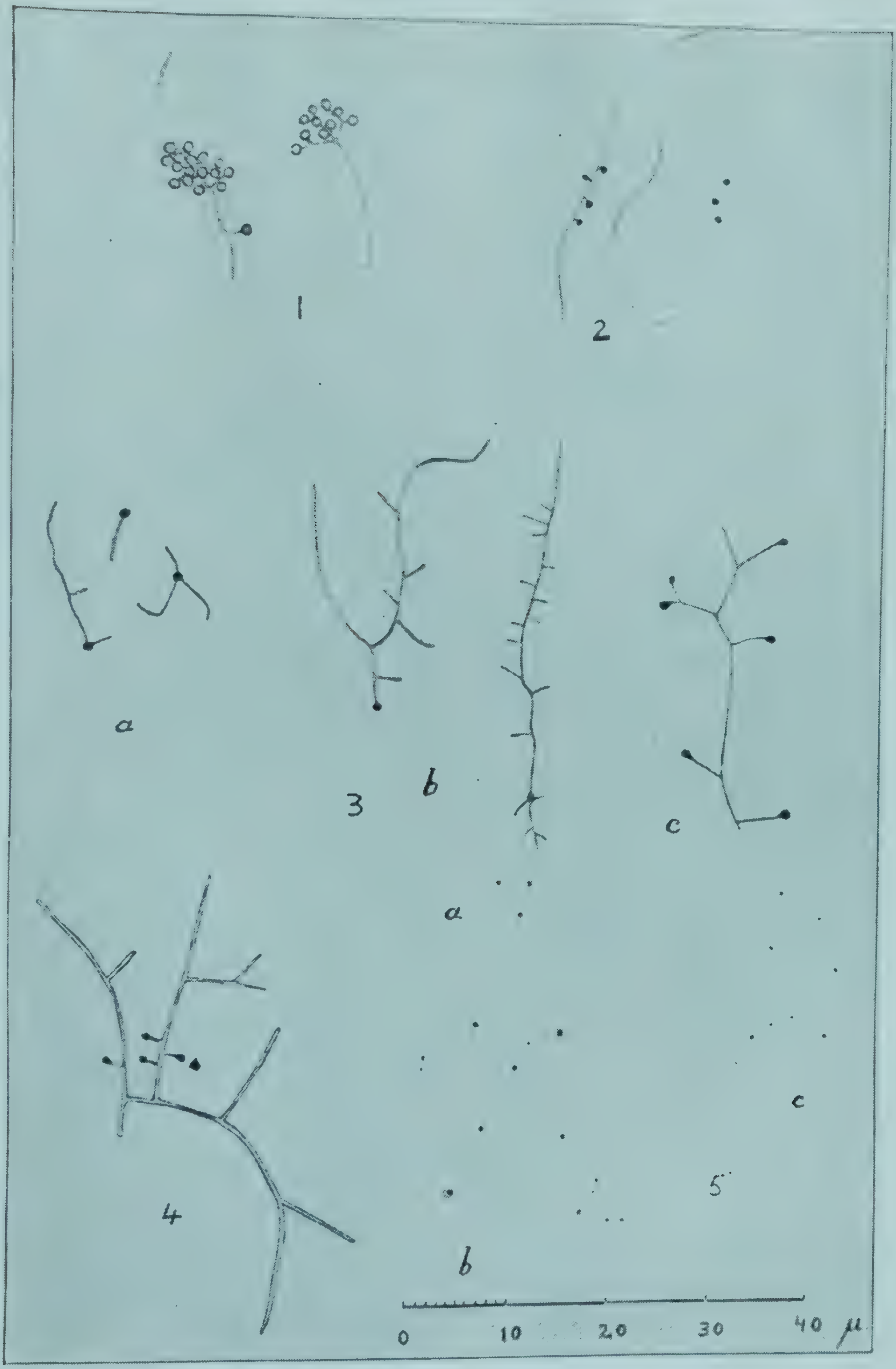


FIGURE 39. Mycelium and sporulation of a micromonospora (Reproduced from: Jensen, H. L. Proc. Linnean Soc. N.S.W. **55:** 249, 1930).

celium in a nondried condition can also be stained with dyes that readily enter the living cell. There is little difference in the staining properties of the various types of mycelium and the aerial spores.

Practically all actinomycetes are gram-positive. Occasionally, a gram-negative form has been reported. The acid-fast properties of actinomycetes, notably among the nocardias, have aroused considerable attention. Numerous acid-fast strains have been described. The microaerophilic and pathogenic species of *Actinomyces* seem devoid of all acid-fastness; so are many species of *No-*

cardia. Others are acid-fast to a variable degree that depends both on the organism and on the cultural conditions.

According to Jensen, acid-fastness in pathogenic forms is often stronger *in vivo* than *in vitro*. Many species can probably best be described as potentially acid-fast, because this property may be apparent for only a very brief period in the organism's life history and can be induced by special media, like milk (Jensen, 1931–1934; Umbreit, 1939) or synthetic media containing paraffin or high concentrations of glycerol (Erickson, 1949).

Acid-fastness is not a permanent property of an organism. On continued cultivation on ordinary media, the acid-fast characteristic may be lost; on the other hand, the property may be strengthened by growing the organism in media containing oil or fat or on animal passage. Acid-fastness cannot be considered as a characteristic for species differentiation.

Some of the observations of stained preparations of actinomycetes have to do with the presence in the thin cells of a nucleus and other particulate constituents. Crystal violet and thionine SO_2, as well as crystal violet-tannic acid-congo red cell wall stain, can be used (Webb and Clark).

Lieske and others failed to observe any true nucleus in actinomycete cells: nuclear substance was found in the form of grains, which, in Lieske's opinion, pointed to the close relationship of these organisms to the bacteria.

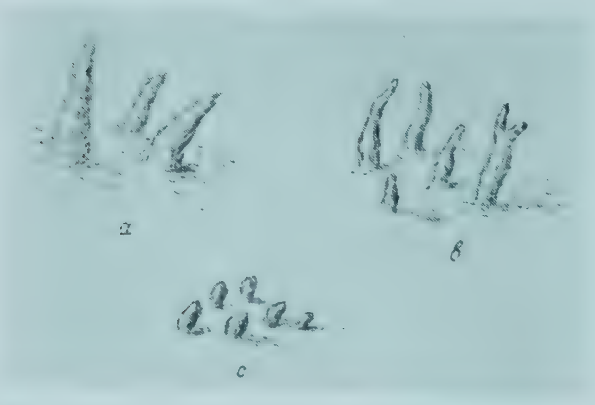


Figure 40. Formation of coremia by a streptomyces (Reproduced from: Krassilnikov, N. A. "Manual of the ray fungi". Acad. Nauk, USSR, Moscow, 1938, p. 34).

The occurrence of fatty particles in the cells of actinomycetes was observed by various investigators. Glycogen and chitin could not be found.

Substrate Mycelium

Actinomycetes produce a substrate or, as it is often designated, vegetative mycelium that usually varies in size, shape, and thickness. The color of the substrate growth ranges from whitish or cream to brownish, yellow, red, pink, orange, green, or black. Water-soluble and water-insoluble pigments may be produced, depending on the organism and the composition of the medium. Some of the pigments, especially the dark or chromogenic pigments, are formed upon complex organic media and are often a result of the action of certain enzymes of the tyrosinase type upon proteins and their derivatives. Other pigments are synthetic in nature and are formed on simple media.

The spores of actinomycetes germinate in the medium with the formation of one or more germ tubes. These grow into long hyphae, finally culminating in a complex mycelium. The length and the diameter of the hyphae differ considerably. Some are straight and reach a length of more than 600 μ; others are only 50 to 100 μ in length and are much branched and curved. This frequently suggested the division of actinomycetes into long-mycelial and short-mycelial groups. The vegetative mycelium varies in diameter from 0.2 to 0.8 μ. The branching of the mycelium is typically monopodial. Involution forms which have a greater diameter may also be produced (Münter, 1916).

On continued growth, the vegetative mycelium becomes brittle and breaks into fragments of uneven length. Some cultures, with age, undergo lysis, others are subject to attack by specific phages. When inoculated into fresh medium, the finer or disintegrated particles give rise to a normal mycelium. This suggested to some investigators (Kober, Monal, Grigorakis) the possibility of symplasm formation as a stage in the life cycle of the organisms. Further study did not substantiate this concept.

Club Formation

An interesting morphological phenomenon among certain actinomycetes, determined by the environment, is the formation of clubs. These clubs should not be confused with involution forms. They are the result not of swelling of the hyphal tip, but of the secretion of a sheath of slime around the tips of the hyphae; therefore they are comparable to the capsules of bacteria. The clubs are formed in the animal body by pathogenic organisms like *A. bovis*. They were also observed by Wright (1905) in cultures growing in the presence of animal serum or whole blood and by Bayne-Jones (1925) in glucose broth. They may be formed in response to the presence of some thermostable substance in animal fluids, or to some other condition such as a reduced oxygen tension. They can be readily induced by the addition of 3 to 7 per cent of NH_4Cl, and also in sugar-containing media on aging. These swellings usually have several times the thickness of the normal hyphae.

In the animal body the clubs are found in groups, each radiating from a mass of mycelium (granules). They give the impression of a star-like arrangement, which is responsible for the name given to the actinomycetes as a whole (Lieske, 1921).

Motility

Motility in actinomycetes was reported for *N. asteroides* by Eppinger (1891) and is characteristic of the genus *Actinoplanes*. Motile organisms were mentioned by Rullman and others. The presence of flagella was never demonstrated, however, except for *Actinoplanes*. Topping (1937) and Ørskov (1938) observed many instances of motility among nocardia-like bacteria from soil. Their strains included acid-fast as well as nonacid-fast forms; they gave both granular and turbid growth in liquid media.

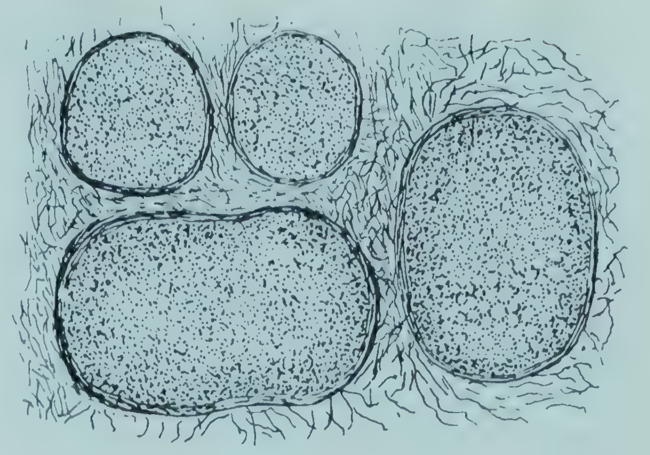


FIGURE 41. Sclerotia formation in a streptomyces (Reproduced from: Thirumalachar, M. J. Nature **176**: 934, 1955).

Jensen examined one of Ørskov's motile strains and found it to agree with *N. citrea*. It produced a soft, lemon-yellow growth on nutrient agar and a diffuse turbidity in broth. The cells were gram-positive but not acid-fast. Direct microscopic examination showed well-developed initial mycelium with mere traces of aerial hyphae. The mycelial structure persisted for a considerable time below the agar surface, but after 24 to 40 hours some of the surface hyphae began to divide into rod-shaped cells that were very strongly motile; this was best seen when a drop of water and a coverslip were placed on top of the agar colonies. In broth cultures, the motility was much less obvious. Staining of the motile cells showed one to four (or more) stout flagella.

Jensen emphasized that while motility may not be common among the nocardias, its existence is indisputable. He suggested the necessity for an alteration in the definition of the order Actinomycetales as constantly nonmotile.

Aerial Mycelium

Species of *Streptomyces* are characterized by the production of a typical aerial mycelium superimposed upon the vegetative growth. The nature of the organisms, composition of medium, and conditions of incubation are of great importance in this connection. Certain actinomycetes have the capacity to form, on agar media, colonies that show concentric rings of sporulating and spore-free zones in their aerial mycelium. One can frequently observe similar zonation in the growth of nonsporulating colonies, the zones being thinner or thicker in the substrate growth of the organisms. Various theories have been proposed to explain this phenomenon, which can be observed also in the growth of certain bacteria and fungi. Among these theories, the following deserve consideration: (a) that it is due to diffusion, by the growing cultures, of metabolites, which stimulate or inhibit the growth of the zone next to that producing the metabolite; (b) that the phenomenon is hereditary in nature. Lieske adhered to the second theory. Too little experimental work has been done to warrant a suitable explanation (Rippel and Witter).

The aerial hyphae may reach a diameter of 1 μ or even 1.4 μ. They vary considerably in length and in structure. The aerial mycelium may cover the whole colony, in the form of a cottony mass or as a powdery, chalk-like surface, or as an almost granular layer.

The sporophores of *Streptomyces* differ greatly in their structure. Some are straight, long or short. The short hyphae correspond to a powdery surface and the long hyphae to a cottony surface. Some of the sporophores are curved, with various degrees of curvature, ranging from very slight at the tip of the sporophore, through "open corkscrew" shaped bodies, finally to compacted spirals, giving a "fist-like" appearance. The sporophores are arranged individually, monopodially, at varying distances from one another, or are close enough together to give a broom-shaped appearance.

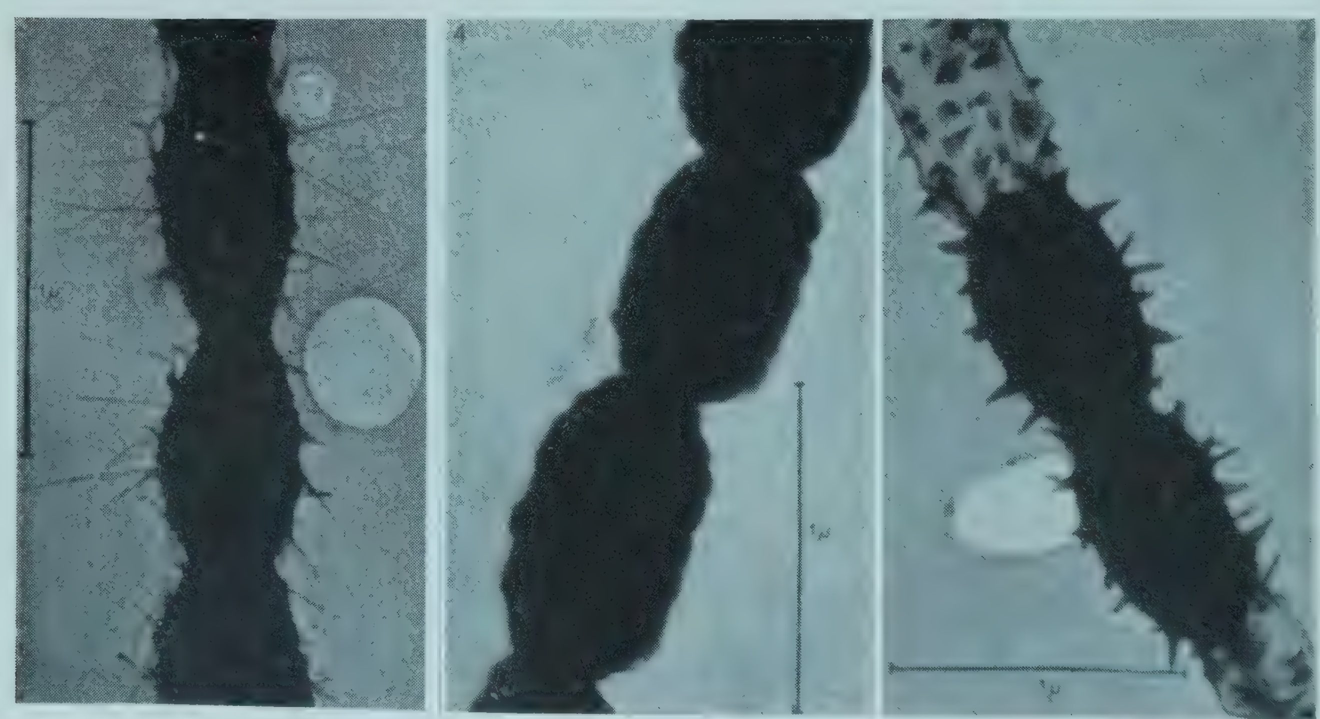


FIGURE 42. Types of streptomyces spores (Reproduced from: Flaig, W. *et al*. Zentr. Bakteriol. Parisitenk. Abt. II. 108: 383, 1955).

The spiral-shaped sporulating hyphae are characteristic in their structure and length, the number of turns being 1 to 20, usually 5 to 10, the curvatures being clockwise (dextrorse) or counterclockwise (sinistrorse). Kutzner examined 382 strains of *Streptomyces*. Of these, 203 formed no spirals, 153 produced sinistrorse and 26 dextrorse spirals, similar to the previous observations of Drechsler and Stapp (1953).

The sporulating hyphae consist of sterile axial filaments bearing sporulating branches in a racemose or capitate arrangement. The primary branches may function directly as sporogenous hyphae or may produce secondary sporulating branches. Sporogenesis is usually confined to the terminal elements, and the hyphal portions below the points of attachment of such branches remain sterile.

The specialized, sporogenous hyphae can be distinguished from the sterile hyphae of the aerial mycelium at an early stage of development. The diameter of the sterile mycelium, which arises through the elongation of the growing hyphal filaments, shows little increase. In the beginning, the sporogenous hyphae are usually thinner than the axial hyphae from which they are derived. After the final linear extension has been attained, the sporogenous hyphae are in most cases appreciably thicker.

The pigmentation of the aerial mycelium is characteristic of the species and depends on the composition of the medium and the length of incubation period. The pigments range from white or gray to yellow, orange, red, rose, lavender, blue and green.

The sporophores in the aerial mycelium of streptomycetes are highly characteristic. Pridham *et al.* (1958) based a system of classification of the genus *Streptomyces* upon this property, as shown in Chapter 20, Volume II. The sporophores are either straight or spiral-forming. As shown previously, the spirals curve not long before the spores are produced; the sporulating hyphae may be curved throughout or only at the end. The number of turns varies in accordance with the length of the spiral, as shown above. Not all the aerial hyphae give rise to spores, some of the hyphae remaining sterile.

Another interesting phenomenon observed in the aerial mycelium of some species of *Streptomyces* is verticil formation, the sporulating hyphae being arranged in groups of 3 to 10, equidistant from one another. Waksman (1919) first reported this for *S. reticuli* and *S. reticuloruber*, the second producing the verticils on the side branches of the aerial mycelium. Kriss (1937), Krassilnikov (1941), Nakazawa (1954–1955), and others attached so much importance to this that the *S. reticuli* group was considered as of considerable taxonomic interest. Some of them produce straight and others spiral-shaped sporulating branches. The composition of the medium is of great significance, synthetic media being most favorable to their formation. Shinobu differentiated between the Nitella type of verticil, in which the sporulating branches are straight (*S. reticuli* type of Waksman), and the Anitella type, in which the sporulating branches are spiral shaped. The "tuft" type of Waksman is considered as a form of the Nitella type in which the distance between nodes is very short. More detailed studies of this have been made by Pridham *et al.* and others.

Prolonged cultivation of actinomycetes may lead to an alteration in the type of aerial mycelium or to complete loss of formation of aerial mycelium. Morphological changes involving similar structures in other microorganisms were found to be influenced by diffusible substances from streptomyces (Grossbard). Dondero and Scotti have shown that old laboratory strains of streptomyces, which had been carried on artificial media for 4 to 40 years, could be induced synergistically to form aerial hyphae again; various strains excreted diffusible substances that caused other strains to form aerial mycelium.

Secondary Aerial Mycelium

Cultures with a normally gray aerial mycelium may be observed to form in spots a white or yellowish mycelium. When such mycelium is transferred to fresh agar, cultures are obtained that differ from the mother culture only in the color of the aerial mycelium but in no other property. Occasionally, a "secondary aerial mycelium," cottony in nature and white in color, is produced from a powdery gray, rose, or blue aerial mycelium. This is usually sterile in nature, as shown, among others, by Jensen (1931) and Kutzner. Cultures obtained from the secondary aerial mycelium (strains) are distinguished from the original cultures (strains) by the color, sterility, and morphology of the aerial hyphae.

Coremia

The sporulating hyphae of certain species of *Streptomyces* may be grouped together into clumps reminiscent of the typical coremia of fungi. They are formed only at certain spots on the surface of the colonies. They were first described by Lieske. The aerial spores of such cultures are no different from those of other cultures. Krassilnikov (1938) reported the formation of coremia both in the substrate and in the aerial mycelium. These coremia are cylindrical or cone-shaped, 1 to 2.5 mm in length, the central portion being made up of vegetative hyphae and the surface of aerial hyphae.

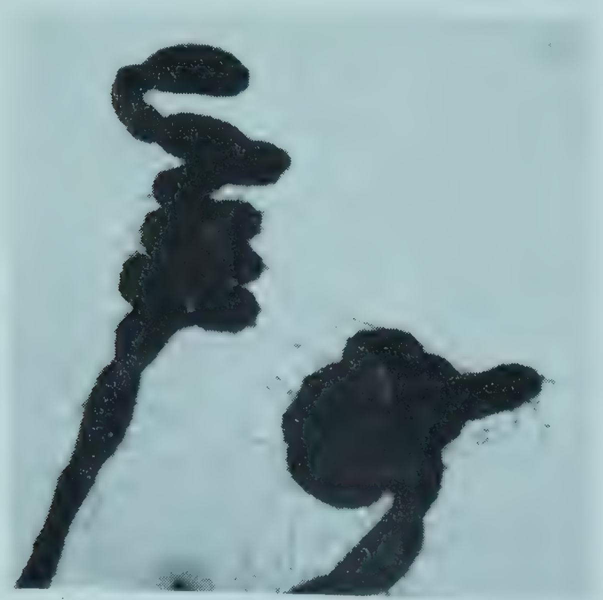


FIGURE 43. Spiral formation by streptomyces (Reproduced from: Baldacci, E. *et al.* Giorn. Microbiol. **1:** 521, 1956).

Spore Formation

As pointed out previously, Lachner-Sandoval was the first to describe, in 1898, the mechanism of sporulation among the actinomycetes. The "fragmentation" spores were considered to be analogous to the spores produced by true fungi. They are formed by the breaking up of the protoplasm within the cell wall into particles or fragments, more or less uniform in size. These are later liberated by the splitting of the cell wall. During the contraction of the fragments, empty and clearer partitions are formed between them, which have been occasionally taken for cross walls. The spore-bearing threads thus assume the appearance of chains of cocci, the spores falling apart readily. This manner of sporulation begins at the top of the aerial spore-bearing hyphae and proceeds toward the base; it is characteristic of the genus *Streptomyces*. Recently, Gordon and Mihm (1958) have shown that certain strains of *Nocardia* (*N. asteroides*) have a similar mode of sporulation in the aerial mycelium.

In the "segmentation" spores, the sporulating hyphae break up by means of cross walls. At first the hyphae are unicellular. At a certain stage of growth, cross walls are produced and the hyphae break up into small segments. These are cylindrical in shape and uniform in size, usually 1 to 2.5 by 0.7 to 0.8 μ. Neukirch (1902) designated the segmentation spores also as "fragmentation spores" and described as "oidiospores" another type of sporulation that appears in older cultures through the break-up of the original hyphae. This manner of sporulation is more characteristic of the genus *Nocardia*.

Drechsler described three types of sporulation in a group of cultures representing primarily the genus *Streptomyces*: (a) true fragmentation, (b) the doubling of the cell wall, (c) contractions similar to segmentation. Duché also recognized three types of spores: (a) regular and irregular arthrospores, (b) microarthrospores, produced in the substrate mycelium, and (c) endospores, produced in the aerial mycelium. True conidia, or "fragmentation" spores, are produced only in the aerial mycelium, whereas the substrate mycelium gives rise to chlamydospores or to arthrospores formed by the concentration of the plasma in the mycelium.

Sporulation among the *Micromonospora* is distinct from that of the other genera of actinomycetes. The mycelium is monopodially branched. The conidia are formed on special branches, which are straight and short (5–10 μ) and which frequently give rise to other branches, thus forming structures similar to bunches of grapes. Each branch bears at the end a single spore, produced by the splitting off of the tip of the hypha. Sporulation occurs most abundantly on synthetic media. Recent studies on sporulation of actinomycetes have been made by Bisset (1957).

Spores

The spores of actinomycetes are spherical (0.3–0.8 μ for *Streptomyces* and 1.0–1.3 μ for *Micromonospora*), oval, or cylindrical (0.8–1 by 0.7 μ for *Streptomyces* and 1.3–1.5 by 1.2 μ for *Micromonospora*). The shape and size of the spores are characteristic of the species. The paired spores for *Waksmania* (*Microbispora*) and *Thermopolyspora* are characteristic for these genera. These spores are reproductive bodies, comparable to fungus spores, rather than resistant bodies, as in the case of bacterial spores. They are destroyed by heat at 60 to 65°C for 10 to 15 minutes. The spores are only slightly more resistant to heat than is the mycelium.

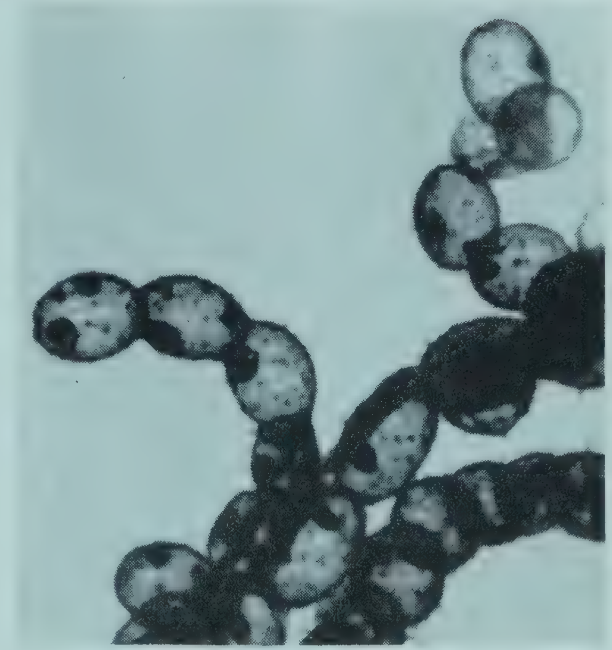


FIGURE 44. Chains of spores (Reproduced from: Baldacci, E. *et al.* Giorn. Microbiol. **1**: 521, 1956).

A detailed study of the nature of the spores of *Streptomyces* has been made by Jensen (1931) and more recently by Flaig *et al.* (1952, 1954). The latter suggested a system of classification of this group of organisms on the basis of spore structure:

I. Spores long, oval-shaped
II. Spores round-shaped:
    1. surface of spores rough:
        a. spiny surface
        b. hairy surface
        c. warty surface
    2. surface of spores smooth
III. Shape of spores cylindrical or rectangular.

On the basis of electron microscopic studies, Flaig *et al.* concluded that these spores are of endogenous origin. They considered the segmentation spores to be produced in the substrate mycelium and as exogenous in nature. Bringmann came to the same conclusions. The composition of the medium rarely has any effect on the form of the spore. Baldacci *et al.* (1955, 1956) observed

TABLE 14

Distribution of different spore types among the various groups of streptomyces (Kutzner)

Group No.	Total number of strains	Total number of subgroups	Strains examined	Subgroups examined	Spores with smooth surface		Spores with spiny surface		Spores with hairy surface		Spores with warty surface	
					Strains	Subgroups	Strains	Subgroups	Strains	Subgroups	Strains	Subgroups
I	374	57	93	17	93	17	—	—	—	—	—	—
II	196	48	47	15	47	15	—	—	—	—	—	—
III	131	37	35	14	33	13	—	—	—	—	2	1
IV	113	25	35	16	22	11	—	—	5	3	8	2
V	56	22	28	12	13	8	13	2	1	1	1	1
VI	441	78	130	34	104	28	25	5	1	1	—	—
VII	432	82	161	48	85	32	28	8	45	6	3	2
VIII	72	25	21	11	18	8	3	3	—	—	—	—
IX	27	4	25	4	—	—	25	4	—	—	—	—
X	14	4	10	4	1	1	8	2	1	1	—	—
Total	1856	382	585	175	416	133	102	24	53	12	14	6

that, in the process of sporulation, an empty spore may appear in a chain of normal spores. This was ascribed to lysis. They also observed giant spores, which were ascribed to anomalies in the septation process.

Kutzner (1956) tabulated a large number of strains of *Streptomyces* for their spore structure, as shown in Table 14. Of the 175 subgroups examined, 133 produced smooth spores, 24 spiny, 12 hairy, and 6 warty spores.

Spores are produced even more readily in streptomyces cultures grown in a submerged state than on the surface of solid or liquid media. The rapidity and extent of sporulation depend entirely upon the strain of the organism. Both kinds of spores are similar in their morphological and physiological properties (Carvajal).

Spores of actinomycetes produced in the aerial mycelium are resistant to desiccation (Berestneff, von Plotho, Stapp). They are highly hydrophylic, with the result that inoculation of stationary liquid media with spores of an organism gives an early surface growth. Erikson (1947) ascribed this to the presence of lipoid substances in their outer wall.

Under favorable conditions of moisture and temperature, the spores of actinomycetes germinate rapidly. They give rise to one to four germ tubes. The different types of spores vary greatly in this respect. The germ tubes may appear at one end or at both ends of the spore. Lieske differentiated between the processes of germination of the spherical and cylindrical spores. Baldacci *et al.* (1956) described several types of germination: single and double unipolar, as well as single and triple bipolar.

Other Reproductive Methods

Reproduction among actinomycetes may also occur by the vegetative process, or through the growth of pieces of mycelium, by the formation of buds which gradually grow into branched hyphal threads, as well as by means of the chlamydospores.

Constancy of Morphological Properties Among Actinomycetes

Although one may observe a certain amount of overlapping among the species of *Actinomyces* and *Nocardia*, and between *Nocardia* and *Streptomyces*, the morphological, chemical, and cultural properties characteristic of each genus justify confidence in their natural status.

A streptomyces may lose, by mutation or by natural variation, the property of forming aerial mycelium; it may thus appear to grow as a typical nocardia. This was found to hold true, for example, of the streptomycin-producing strain of *S. griseus*. When this occurs, it is accompanied by a change in the physiology of the organism, but not in the morphological properties of the vegetative growth. Frequently such a culture may revert to its original form and produce typical aerial mycelium.

To retain the property of proper sporulation, cultures are usually maintained in soil. A good soil with a fair supply of organic matter, neutral or slightly alkaline in reaction, with a moisture content of about 60 per cent of the water-holding capacity so as to favor proper aeration is inoculated with a spore suspension or with substrate mycelium. The flask is incubated at optimum temperature until good growth is obtained. The flask is then thoroughly shaken and further incubated. After growth has reached a maximum, the soil is allowed to desiccate. When the culture is then transferred from the soil to a synthetic agar medium, good sporulation is obtained. Excess organic nitrogenous nutrients, especially phospholipids, favor substrate growth at the expense of the aerial mycelium, as shown by Erikson (1947).

Morphological Characteristics of Some of the Important Genera of Actinomycetes

Actinomyces

The anaerobic pathogenic organisms belonging to the genus *Actinomyces* are gram-positive, nonacid-fast, producing a branching vegetative mycelium. Nonsporing aerial hyphae are occasionally formed in the firm-textured *A. israeli*. *A. bovis*, however, produces soft, smooth colonies, without aerial mycelium. The vegetative mycelium divides into diphtheroid rods (Erikson, 1949). Club formations in tissues of the host, which attracted so much attention among earlier workers and which were also found (Wright, Naeslund) in serum or ascitic fluid media, are now recognized to be mechanisms of resistance of the host against invasion.

According to Erikson (1953), the combined demands of anaerobiosis, parasitism, and complex nutritive requirements apparently leave little scope for mycelial variation among the members of the genus *Actinomyces*. These organisms exhibit a pattern of development similar to that of the nocardias, and the chief type of variation reported is that of "smooth" and "rough" colonies. The former is associated with readily fragmented growths producing turbidity in liquid cultures; the latter with coherent, filamentous, well-branched growths leaving the liquid substrate clear in the manner characteristic of actinomycetes in general. The changes described are from the typical, rough, breadcrumb colony to the soft smooth one, not the reverse (Triuss and Politowa, 1931; Lentze, 1938; Ludwig and Sullivan, 1952).

According to Morris, *A. bovis* passes through a complete life cycle, consisting of two clearly defined generations. The germination of the spores takes place by budding. The mycelium, if formed, is less stable than in the case of streptomyces. Branching is impermanent in the haploid phase, but is permanent in the diploid phase. The mechanism for the formation of the initial cell in the genus *Actinomyces* is less specialized than in the genus *Streptomyces*. A haploid generation gives rise to a diploid generation by conjugation of two specialized haploid cells. The diploid generation produces a haploid spore by reduction division. The club shape of the newly germinated diploid form and the altered shape of the haploid phase cells represent the pleomorphic forms described by various investigators in certain specialized media, such as those containing a high concentration of serum.

Various reports concerning filterable forms of actinomycetes are found in the literature. It is sufficient to cite a recent contribution by Monal. Cultures of *A. bovis* were filtered through a collodion membrane. When the filtrate was supplemented with various nutrients, growth took place. This consisted of filamentous forms and typical arthrospores; there were also a "symplastic stage," diphtheroid forms, streptococcal forms, and a type of cell similar to a T or a double Y. Suspensions of *A. bovis* obtained from culture collections or isolated from human cases were placed in collodion sacs, which were introduced into the peritoneum of rabbits. After the first and second passage, the animals showed no symptoms of actinomycosis. After the third passage, consisting in the inoculation of the rabbit with the product of the second passage, atypical actinomycosis was produced, in which the parasite was in the form of a symplastic or of a diphtheroid bacillus. After the fourth passage, typical actinomycosis was obtained. The conclusion was reached that cultures of *A. bovis* contain growth elements that will pass through a collodion ultrafilter. Erikson (1940) did not confirm the ability of actinomyces to give rise to filterable forms.

Erikson emphasized the difficulties encountered in isolating an actinomyces from the mixed flora obtaining in most pathological material; the low fertility rate of dismembered mycelial fragments and individual cells; and the need for anaerobic methods of cultivation. Successful isolation depends to a high degree on fresh material containing a quantity of viable elements. Although Erikson stated that her experiments "have disposed of the existence of a filterable stage and have yielded no evidence in favor of any hypothetical life cycle involving either anaerobic or aerobic forms," Kriss *et al.* (1945) stated that the hyphae of actinomycetes in a single colony may be so thin as to lie beyond the limits of the visible light microscope and may thus yield filterable forms. More recent studies on the sporulation process of *Actinomyces* have been made by Bisset, as shown later (Vol. II, Chapter 24).

Nocardia

According to Jensen, nocardias are characterized by the early formation (when isolated cells are allowed to develop into microcolonies) of an initial mycelium that sooner or later divides into irregular rod-shaped cells resembling mycobacteria or corynebacteria and often becoming so short as to look like cocci. The initial mycelium varies in extent from hardly visible, irregular, elongated rods with a few short lateral branches, to something approaching that of *Streptomyces*, from which it differs only by a more loose and crumbly consistency when the hyphae begin to divide. Swollen cells that on fresh medium germinate with one or more slender "germ-tubes" are of common occurrence. The aerial mycelium of *Nocardia* varies to a similar extent. It is often invisible to the naked eye, and may be altogether absent or may consist of a few short filaments, which sometimes look like mere granules. At the other extreme, the aerial mycelium may be abundant but composed of elements resembling those of the vegetative mycelium, usually less branched than in *Streptomyces*, and usually not differentiated into conidia-like spores.

The nocardias are related, on the one hand, to the mycobacteria, and on the other, to streptomyces. This gradual transition from one group of microbes to another must, in the words of Erikson (1945), "be accepted as another of the innumerable instances in which nature prodigally overlaps manmade taxonomic boundaries." In the early stages of development, nocardias are characterized by the formation of an undivided substrate

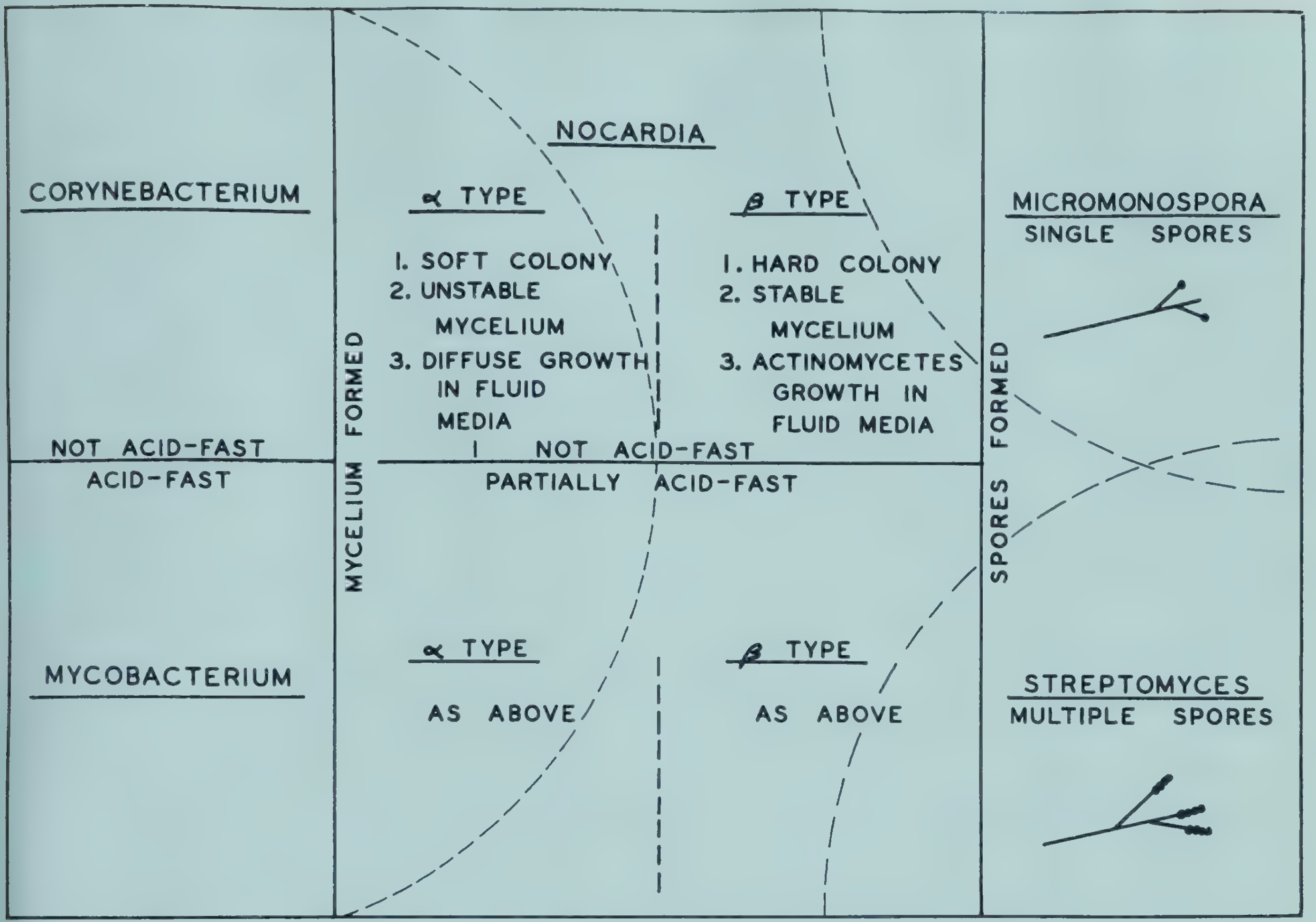

NOCARDIA
CORYNEBACTERIUM
NOT ACID-FAST
ACID-FAST
MYCOBACTERIUM
MYCELIUM FORMED
α TYPE
1. SOFT COLONY
2. UNSTABLE MYCELIUM
3. DIFFUSE GROWTH IN FLUID MEDIA
β TYPE
1. HARD COLONY
2. STABLE MYCELIUM
3. ACTINOMYCETES GROWTH IN FLUID MEDIA
NOT ACID-FAST
PARTIALLY ACID-FAST
α TYPE
AS ABOVE
β TYPE
AS ABOVE
SPORES FORMED
MICROMONOSPORA
SINGLE SPORES
STREPTOMYCES
MULTIPLE SPORES

FIGURE 45. Relationships of the genus *Nocardia* to other genera of actinomycetes (Redrawn from: Umbreit, W. W. and McCoy, E. *Symp. on hydrobiology*, p. 108, 1941).

mycelium. Aerial hyphae may be formed, but they are usually indistinguishable from the substrate mycelium. The nonseptated hyphae of both the substrate and aerial mycelium break up into short rods and cocci, by the "segmentation" process, giving rise to oidia-like spores. These germinate, forming a true mycelium. Nocardias are either acid-fast or nonacid-fast. Some nocardias are characterized by marked pleomorphism (Karwacki). The angular type of growth described for some of the actinomycetes is also a property of some nocardias.

Erikson (1949) examined 300 strains of nocardias, some freshly isolated and some obtained from culture collections. On immediate isolation, only 9 per cent were partly acid-fast, but on subsequent cultivation on media rich in organic matter, such as milk or nutrient broth, this percentage increased to 31. These nocardia cultures ranged from soft mycobacterial type growth, with transient vegetative mycelium and very sparse aerial mycelium, to the harder streptomyces-like forms. No evidence was obtained of any resting spores or chlamydospores in the vegetative mycelium. The lack of aerial spores, as well, led to the conclusion that the nocardias should be regarded as asporogenous. The phenomena of "cystites" of Jensen (1932) and "involution forms" of Ørskov were believed to be vegetative in origin.

Ørskov divided the nocardias into two groups: IIa, with aerial mycelium, and IIb, without aerial mycelium. The first is associated with a more stable, well-branched vegetative mycelium approaching in structure that of the streptomyces. The second produces soft, mycobacterial, unstable mycelium. According to Krassilnikov (1938) the scant aerial mycelium of the first group is devoid of branches and spirals and divides into sharp-ended cylindrical cells. Reference has already been made to the recent observations of Gordon and Mihm (1958) on the sporulation of *N. asteroides* in a manner similar to that of streptomyces.

Jensen (1931) described filamentous and short-celled forms of the saprophytic *N. polychromogenes*, as pointed out in Chapter 6. Umbreit (1939) also distinguished several distinct types among the nocardias: α-forms with short unstable mycelium, soft colonies, and diffuse growth in broth; β-forms with long stable mycelium, firm streptomyces-like growth on agar, and colony type of growth in liquid media, which remains clear. Umbreit considered the growth in broth a more definitive characteristic than any other, although he remarked that "borderline cases exist in which the investigator must resort to other characteristics." Jensen suggested division of the genus *Nocardia* into two genera, reserving the last name for the β-forms and retaining the name *Proactinomyces* for the α-forms, but he added that these names could probably with equal right be used for the potentially acid-fast and the nonacid-fast strains, respectively. A horizontal division (β-forms = *Nocardia*, α-forms = *Proactinomyces*) would give considerable morphological homogeneity, except in respect to motility, but would entail much heterogeneity in respect to potential acid-fastness and the correlated characters of carbon metabolism and proteolytic power. On the other hand a vertical division (acid-fast = *Nocardia*, nonacid-fast = *Proactinomyces)* would result in greater biochemical homogeneity but a corresponding morphological heterogeneity. The relationship of these nocadial forms to the bacteria, on the one hand, and to the streptomyces and micromonospora on the other, is brought out in Table 15.

McClung (1949) observed that if fragmentation follows very shortly after germination, branching will be sparse and very little mycelium will be developed. With a delay in fragmentation, there is opportunity for repeated branching; a larger or smaller mycelium may result, according to the rate of growth and the amount of subsequent cell division. The time of branching and fragmentation in a given strain was relatively constant under controlled conditions.

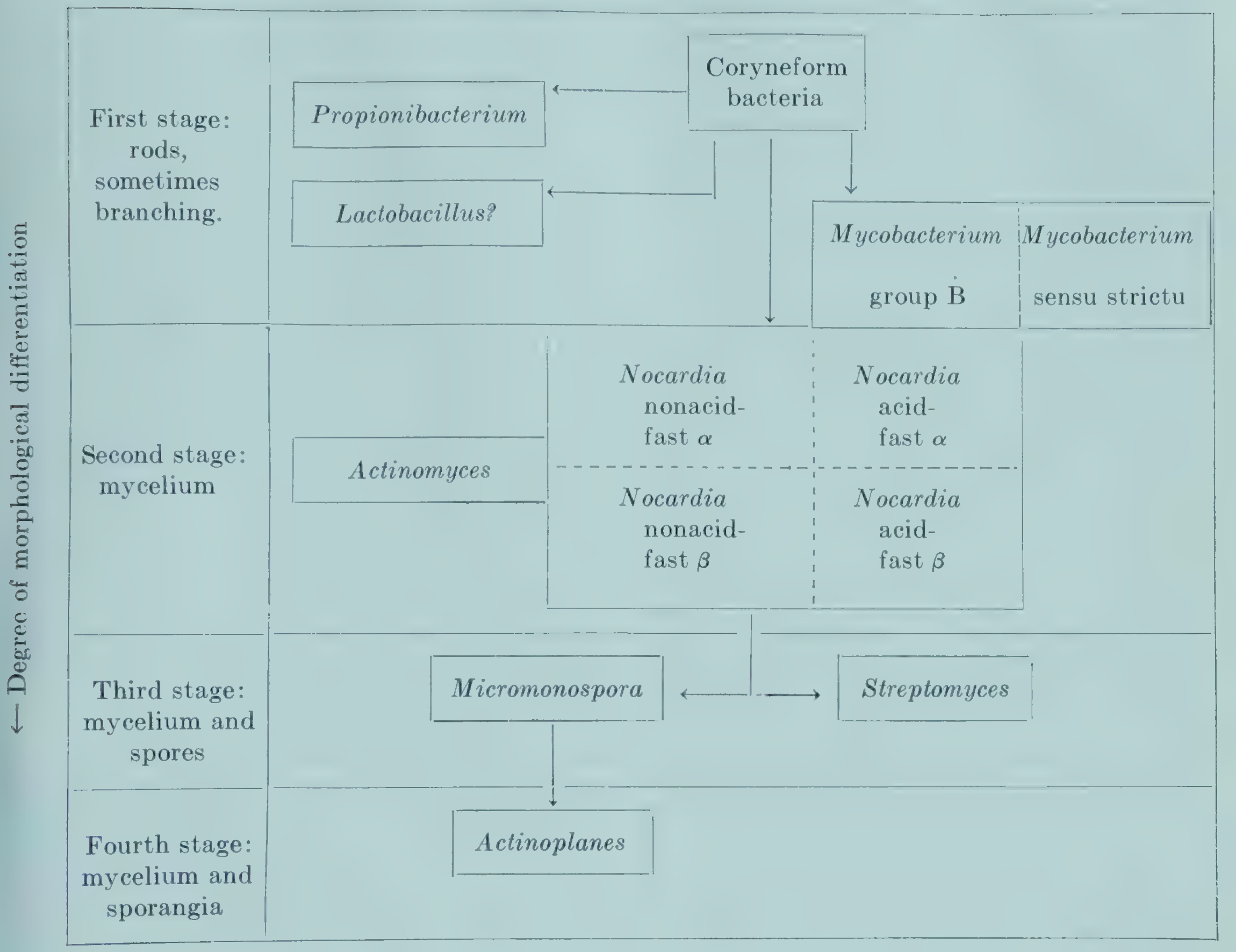
McClung divided the nocardia group into three subgroups:

I. Scant mycelial development, sparse branching, and type 1 fragmentation. Colonial texture soft, pasty and sometimes mucoid, pigment intracellular and insoluble.
II. Extensive mycelial development, straight branches which do not overlap, and type 3 fragmentation. Colonial texture soft and pasty, pigment intracellular and insoluble.
III. Extensive mycelial development, no fragmentation of hyphae, contorted and profusely produced branches which overlap. Colonial texture waxy or cartilaginous. Generally both intracellular and soluble pigments are produced.

Morris describes as follows the life cycle of *Nocardia*: At first, a microcyst is formed, which germinates on budding. The bud gives rise to a long multinucleate filament, which divides into individual cells by the formation of transverse cell walls. The cells multiply by simple fission, by complex vegetative reproduction, and by branching. No prolonged diploid phase, similar to that of actinomyces, is produced. Heat-fixed gram-stained preparations of nocardia and actinomyces may resemble each other, but cytologically they are markedly different. The conclusion was reached that there can be no doubt that the aerobic nocardia constitutes a separate genus from the anaerobic actinomyces. The germination of the microcyst of nocardia by budding is comparable to that of the spore in streptomyces, in micromonospora, and in actinomyces, but is different

TABLE 15

Tentative phylogenetic scheme of the Actinomycetales (Jensen 1953)


← Degree of morphological differentiation
First stage: rods, sometimes branching.
Second stage: mycelium
Third stage: mycelium and spores
Fourth stage: mycelium and sporangia
Coryneform bacteria
Propionibacterium
Lactobacillus?
Mycobacterium group B
Mycobacterium sensu strictu
Actinomyces
Nocardia nonacid-fast α
Nocardia acid-fast α
Nocardia nonacid-fast β
Nocardia acid-fast β
Micromonospora
Streptomyces
Actinoplanes

from that of Eubacteriales. The important difference between actinomyces and nocardia consists in the production by the former of true reproductive spores and the occurrence of a prolonged diploid phase, in contrast to the occurrence, in the latter, of a microcyst and a greatly reduced diploid phase. When branching occurs in nocardia, it is not of the permanent type but resembles that of an actinomyces, and is thus differentiated from permanent branching. Both genera have a well-marked haploid generation arising from a spore by budding, and a diploid one arising from the initial cell.

Webb and Clark (1957) demonstrated the existence in the growth cycle of *N. corallina* of a unicellular, uninuclear coccoidal stage. By proper staining techniques, they observed nuclear structures comparable to the nuclei of higher organisms. Fragmentation of the hyphae was preceded by cross-wall formation, the first step of fragmentation being the reorganization of the hyphal nuclei. The formation of the cross walls was preceded by nuclear division.

Further information on the early studies of the nocardia group, their relationship to the mycobacteria, and their morphological changes is found in the work of Vierling, Karwacki, Badian, Bisset, Hagedorn, and numerous others. The life cycles of nocardias have been discussed further by Combes *et al.* (1957).

Streptomyces

This genus is characterized by the permanently undivided character of its substrate mycelium and by the formation of spores in its aerial mycelium. Instances do occur, however, in which the ability to form aerial mycelium is lost by mutation (Erikson, 1948), and also in which organisms with the typical spore-apparatus of streptomyces produce a soft-textured, easily fragmenting substrate mycelium, and even turbidity in liquid media (Jensen, 1931; Ørskov, 1938). If such strains should permanently lose their ability to form aerial mycelium, they would obviously become indistinguishable from true *Nocardia*. Wright (1937) described the formation of corynebacterium-like variants in streptomyces. Jones (1949) also described such strains, of which the classification seems arbitrary.

Erikson considered the streptomyces to be among the most successful of all microorganisms. To have attained such universal distribution, it is clear that the organism itself (the genotype) must possess a balance of favorable characters. She described the most important of these as follows: 1. The reproductive rate and efficiency of its mechanism. 2. The duration of life of the organism. 3. The resistance of the organism to exterior influences. The most valuable property is that of producing large numbers of special, resistant cells (spores). This is the great advantage possessed by the genus *Streptomyces* over the genus *Nocardia*.

The substrate mycelium of streptomyces develops homogeneously, giving rise to a tough-textured, cartilaginous growth, with a smooth or rough and lichenoid surface; it tends to adhere strongly to the medium. The substrate mycelium does not divide during the course of development, and gives rise to a somewhat thicker aerial mycelium. The latter is formed expecially on synthetic media. The aerial hyphae produce straight or curved sporulating branches, giving rise to conidia, by the process of "fragmentation." The process of "segmentation," or oidia formation, may also occur in the vegetative mycelium (Jensen, 1931; von Plotho, 1940). The substrate mycelium may produce chlamydospores.

According to Erikson (1953), the formation by streptomyces of a thick, glossy, tough growth, completely devoid of aerial mycelium, that adheres so closely to media represents a common instance of physiological adaptation. This vegetative growth may occur on many substrates rich in nitrogen and in phosphorus. These variations may be temporary, the characteristic pattern of growth being restored by returning the organism to a suitable, better balanced, and simpler medium; sterile soil is quite suitable.

Erikson (1948, 1953) noted further that the absence of readily fermentable carbohydrates in the medium resulted in a marked lowering of the variability rate when cultures of streptomyces were plated out on similar media containing glucose, sucrose, starch, lactate, or acetate. The toxic conditions arising in unaerated or solid cultures were said to be a result of the accumulation of organic acids following the dissimilation of sugars: "The secondary colonies that arise at the periphery of established growths on old plates frequently exhibit a distorted and shrunken appearance, with straight aerial filaments instead of spirals, colorless or only faintly pigmented, and with unusual modes of vegetative branching. Such cases of unstable reversible modifications commonly make up the greater part of the heterogeneity of streptomycetes capable of synthesizing complicated structures from minimal media."

Drechsler considered the actinomycete mycelium to be definitely septated, the hyphae being divided into short sections. This phenomenon is particularly striking in cultures belonging to the nocardias, but appears only seldom among the streptomycetes. Ørskov and others believed that formation of septa is the first stage in the process of the break-up of the mycelium into fragments.

Certain swellings of the terminal ends of hyphae may be observed in old cultures. They are also formed under abnormal growth conditions, as in concentrated media or in the presence of certain specific substances like caffeine. These swellings may be considered as involution forms, somewhat similar to the clubs produced by pathogenic actinomycetes in the animal body.

Further information on the morphology of the streptomyces group is found in the work of Knaysi, McGregor, Penau *et al.*, and Prokofieva *et al.* The degeneration of streptomyces was analyzed by Williams and McCoy (1953). See also studies by Bisset (1957).

Micromonospora

Members of the genus *Micromonospora* are characterized by the formation of a well-developed branching vegetative mycelium, similar to that of *Streptomyces.* Single spherical to oval spores are produced on the tip of special sporophores or side branches of the vegetative mycelium. No surface growth is formed in liquid media. When such media are stirred or continuously shaken, thus resulting in the breaking up of the spores, an abundance of new clumps or colonies are produced. Aerial mycelium, if formed at all, is of the nocardial type, consisting of filaments mostly unbranched and undivided. Micromonosporas have frequently been looked upon as the most highly developed group of actinomycetes, closest to the fungi.

Erikson (1953) described *M. chalceae* as producing a slowly developing mycelium of very slender, profusely branched filaments, bearing single spores on short lateral branches. There is no aerial mycelium; in instances where it has been reported, it is sparse, infrequent, and reversible. This investigator emphasized the profound physiological differences between the mesophilic micromonosporas which can utilize cellulose, chitin, lignin, and other resistant compounds, and the thermophilic forms, which cannot (Erikson, 1941, 1952). The thermophilic *M. vulgaris* was said to exhibit consistently a pattern of mycelial development which differs unmistakably from that of the mesophilic *M. fusca.* Pigment was not produced by the thermophilic species.

According to Erikson (1953), the most characteristic morphological properties of the thermophilic *M. vulgaris* is that it forms at 60°C an abundant white felt of aerial mycelium. The filaments of the secondary aerial mycelium bear single, highly refractile spores on short lateral branches, in the same manner as do the vegetative filaments of the primary mycelium. Since only sparse development of aerial mycelium takes place at 37°, this secondary aerial growth was regarded as an expression of thermal dimorphism. It plays an important part in the life of the organism, as indicated by the high oxygen uptake by the aerial mycelium at 60°, as compared with the very low values given by the primary vegetative growth.

The aerial mycelium of *M. vulgaris* segments rapidly to form elongated branching chains of short cells which "bud," producing spores on very short stalks. The vegetative mycelium continues to show undifferentiated filaments which sooner or later disintegrate, thus showing a much lower degree of viability. These properties also can be demonstrated in the mesophilic micromonosporas. Very little vegetative growth of *M. vulgaris* is produced on artificial media and in its natural habitat. There the proportion of aerial mycelium and spores is visibly much higher. These results tend to support the decision made by Henssen and in this treatise to separate the thermophilic and mesophilic forms into different genera.

The life cycle of *Micromonospora* was described by Morris as follows: The spore germinates by budding and the bud develops into a long filamentous cell, which divides into a number of individual cells by the formation of transverse cell walls. The branches are permanent, since no transverse wall is produced at the junction of the parent cell and the branch. A haploid mycelium is thus developed; the filamentous outgrowths from the cell show a direct protoplasmic connection between cell and filament. Where two filaments make contact, an ovoid swelling appears, forming a complete cell, eventually enclosed in a cell wall. These were believed to be diploid. These cells give rise, on germination, to a "secondary mycelium," multicellular in nature with permanent branching. Single spores arise at the end of their stalks, formed at first as finger-like protrusions of the mycelium.

The morphological features of the other genera of actinomycetes are discussed in Chapters 26 to 29 (Volume II).

CHAPTER 6

Variations, Mutations, and Adaptations

Concepts of Constancy of Characters

An actinomycete culture is made up of an extensive mass of mycelium, either of a substrate or vegetative nature or of both substrate and aerial hyphae. Sooner or later, the mycelium breaks up into a large number of different kinds of cells and spores. These vary greatly in size and in shape, and frequently in certain physiological properties. Largely because of this, as well as under the influence of different environmental and nutritional factors, cultures originating from the various cells and spores give rise to strains that may show quite distinct variations from the original culture. These variations may involve colony structure, formation of soluble and insoluble pigments, sugar utilization, virulence, antibiotic production, and resistance to antimicrobial agents.

Following the concept of Ferdinand Cohn and Robert Koch, many, if not most, bacteriologists once considered the bacterial cell as constant in nature and immutable or monomorphic. This attitude tended to discourage investigations of problems of variation and inheritance among microorganisms. On the other hand, the pleomorphists were inclined to consider the microbial cells as undergoing considerable metamorphosis and constantly giving rise to new species. The history of microbiology is replete with the changing influence of these two schools, one concept gaining the upper hand at one time, and the other at another time.

Recently, the old problem of morphologic variation among bacteria has been reopened to an extent that many of the modern pleomorphists tend to return to the older concepts of Naegeli and other earlier pleomorphists that any bacterium may transmute to form any other bacterium.

Enderlein coined a vocabulary of nearly two hundred new words to express his ideas. The life cycle of a bacterium was said to consist of two simultaneous, parallel, and coordinated processes: (a) a multiplicative development through simple cell division, and (b) a progressive development, very slow and characterized by morphologic variation. It was believed that as the ontogeny of an individual repeats the phylogeny of the race, so does the life cycle of a bacterium repeat the evolution of the species; the bacteria were said to be derived from and to return to an elementary unit, the mychit. The life cycle of bacteria was said to begin with the fusion of two hapioid mychits, followed by progressive changes in cell complexity; when a maximum, fixed for the species, is reached, there is a final return to the haploid mychit. The cycles may be incomplete, shortened, or completely arrested.

This confusion was also reflected in the literature on the cyclogenie of actinomycetes. It is sufficient to cite the findings of Nepomnjaschy, who recognized, among the variants of an actinomycete isolated from the pus of patients, three types of dissociation: 1. S-type—smooth, transparent colonies, consisting of gram-negative rods, growing an-

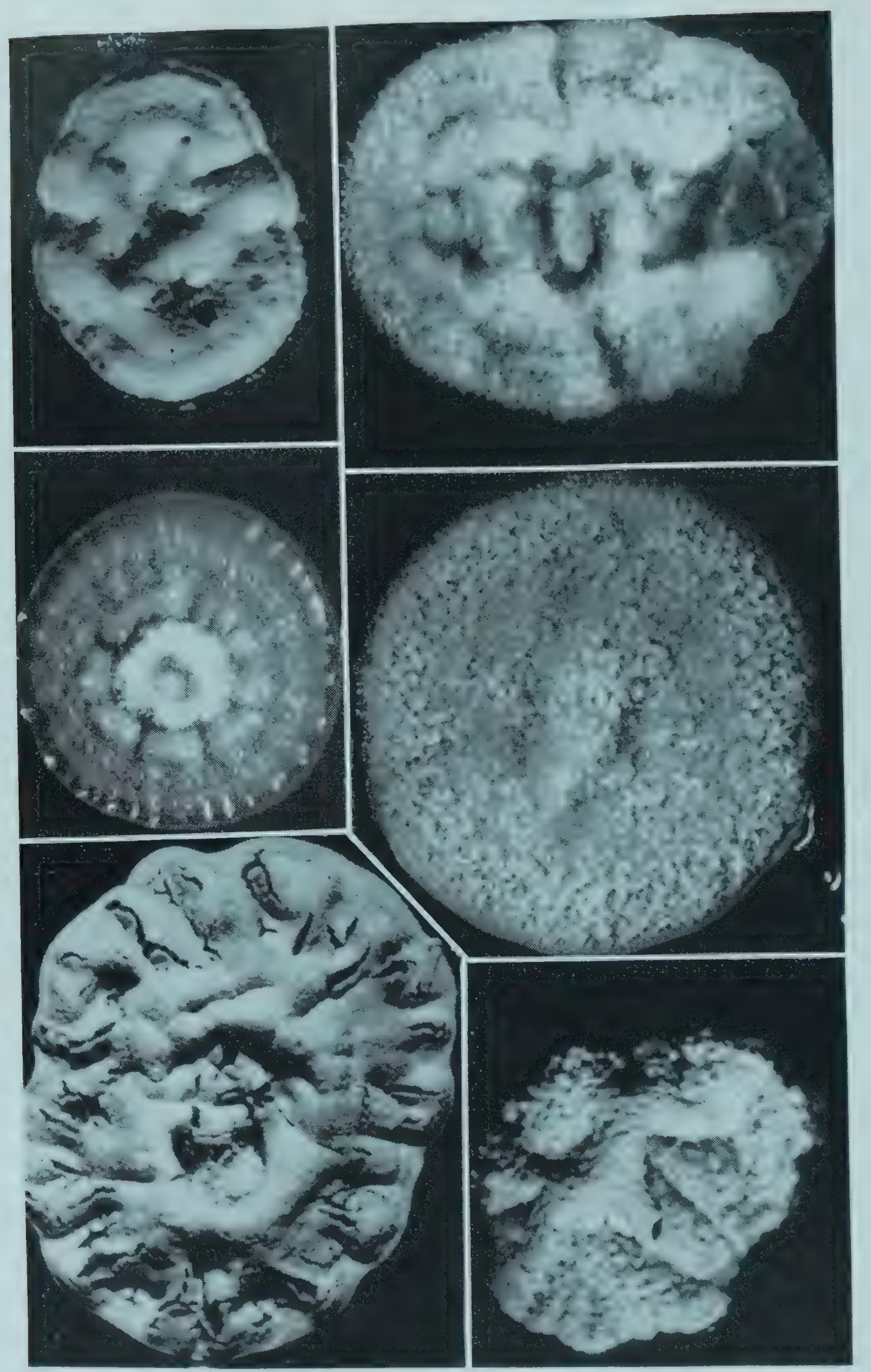


FIGURE 46. Colony variants of *S. griseus* (Reproduced from: Dulaney, E. L. *et al.* Mycologia **41:** 390, 1949).

aerobically and nonpathogenic. 2. O-type—gram-positive, diphtheria-like rods, considered as anaerobic transition forms, capable of developing in the human organism. 3. R-type—large colonies, made up of aerobic, gram-positive rods. This cyclic development was said to be accompanied b changes in the cultural, morphological, an biochemical properties of the organism.

Henrici, however, emphasized that th

concepts of the pleomorphists showed a complete lack of systematic investigation. In an attempt to patch together the life cycles from haphazard observations of cultures in widely different media, no consideration was given to the age of the culture or to the phase of growth. The designation of all the structures by names borrowed in part from mycology, in part from cytology, or coined for the occasion served only to obscure the problem and to make it more difficult for the reader to follow. Henrici emphasized that the necessity for creating a new terminology had its origin in the obscurity of the thought which the author was trying to express. The confusion introduced by this terminology was all the greater because the same structures were referred to by the various authors under different names, and the same names were used to designate different structures. Henrici demonstrated experimentally that bacteria vary continually in morphology with increasing age of the culture. He correlated morphologic variations with the rate of growth, and showed that the transition from one type to another occurs at the points of inflection between phases of the growth curve.

Variations Among Microorganisms

Among the major factors that influence variability of microorganisms are: (a) the previous history of the culture, (b) the nature of the substrate or nutritional conditions, and (c) the environmental factors. Different organisms differ in this respect: some species greatly resist variation, others readily undergo variation.

One must differentiate between gradual variation of an organism growing in a certain medium and mutation of the organism that consists in a complete change of one or more characters. Mutations are represented by the appearance or disappearance of certain morphological or physiological properties, including production of pigment, formation of specific enzymes, power to cause infection, production of aerial mycelium, and manner of sporulation.

Some of the variations obtained for certain organisms may be permanent in nature; others are only temporary. When a culture is so treated as to result in injurious or stimulating effects, some of its properties may be lost, whereas others may be gained. Certain characteristics of the culture may not change as a whole, but undergo only a degree of change, as in the formation of adaptive enzymes. Such variations are usually quantitative rather than qualitative in nature. When a comparison was made of the properties of a number of strains of *S. griseus*, whether isolated from different substrates or obtained from a single culture, all degrees of gradation were obtained. These variations involved the ability of the strains to produce antibiotics or enzymes, the intensity and nature of soluble and insoluble pigments, and the length of the aerial hyphae.

The effect of temperature upon the behavior of a given culture is illustrative. It is sufficient to cite the classical observations of Pasteur concerning a change in pathogenicity of the anthrax organism when cultivated at 42°C, and the loss by *Serratia* grown at 37°C of its power to produce its characteristic pigment. Other illustrations comprise the increased power of infection brought about by the reisolation of an organism from animal tissues infected with it.

Variations among microorganisms include the following:

1. Nonhereditary modifications brought about by the unequal influence of different conditions.

2. Hereditary continuous variations, characterized by the gradualness of the change through successive generations. These include (a) adaptive variations, in which the nature and direction of the change bear an adaptive relationship to the conditions under which the change appears, (b) nonadaptive

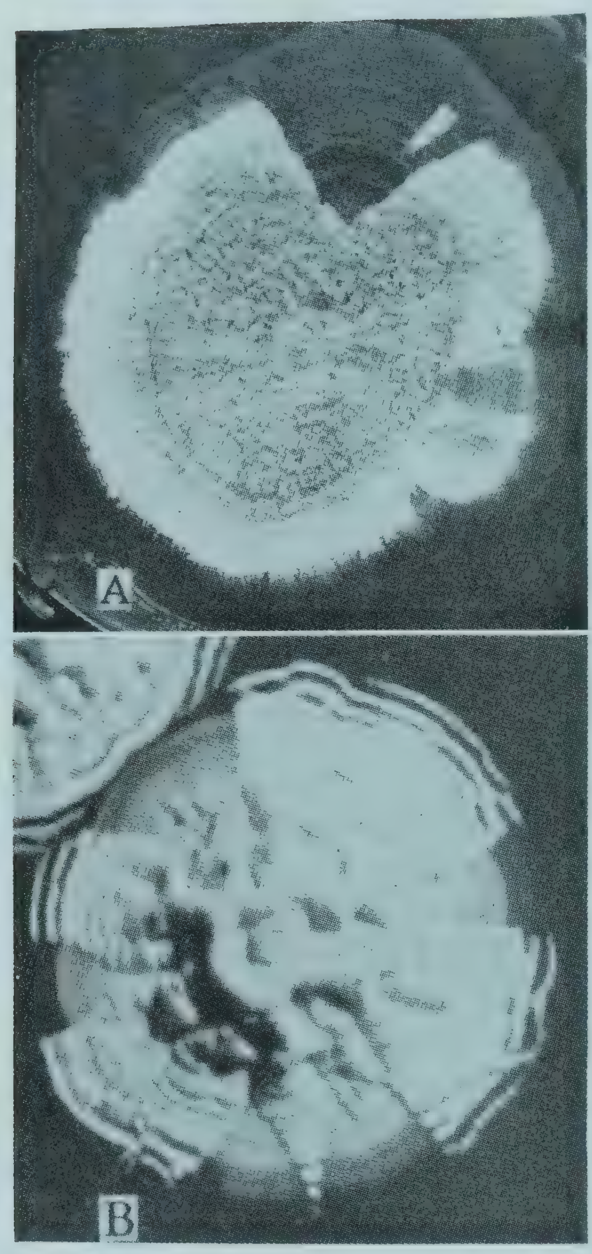


FIGURE 47. Colony sectoring in *S. griseus* (Reproduced from: Carvajal, F. Mycologia 38: 603, 1946).

variations, in which the change bears no apparent adaptive relationship to the conditions under which the change appears. Both may be reverting and nonreverting variations.

3. Hereditary discontinuous variations, characterized by the suddenness of their appearance. These, as well, include adaptive and nonadaptive conditioned variations. A character appears under certain conditions, and either bears or does not bear any adaptive relationship to those conditions. These variations may also be reverting and nonreverting.

All these variations find their counterpart in the case of actinomycetes.

Variations Among Actinomycetes

The extreme variations that were observed for actinomycetes led some of the early workers to become greatly discouraged in their attempts to recognize species. This was succinctly expressed by Henrici, who said, "It is largely due to this variability that our knowledge of the species of actinomycetes is so uncertain and more or less chaotic."

In spite of these variations, the constancy of strains or species of actinomycetes can be maintained if proper care is taken in growing the cultures on suitable media. The recognition of this fact has led some investigators, notably Ørskov and Erikson, to emphasize the constancy of the characters of actinomycetes.

Early students of the actinomycetes reported the fact that variations among these filamentous organisms are of several distinct types. Lieske emphasized that actinomycetes show greater variability in their morphological and physiological properties than do any other group of microorganisms. The types of variation were classified by him into: (a) simple modifications, (b) permanent modifications, and (c) mutations. Under the influence of various environmental conditions and on continued cultivation, actinomycetes undergo both quantitative and qualitative variations. Certain pigments may be lost entirely or changed in nature or intensity. The property of forming aerial mycelium may be either lost or regained. The size, shape, and color of the colonies, the length and abundance of the aerial mycelium, and the manner of spore formation may all vary.

Variations among actinomycetes can also be divided into: (a) adaptive variations,

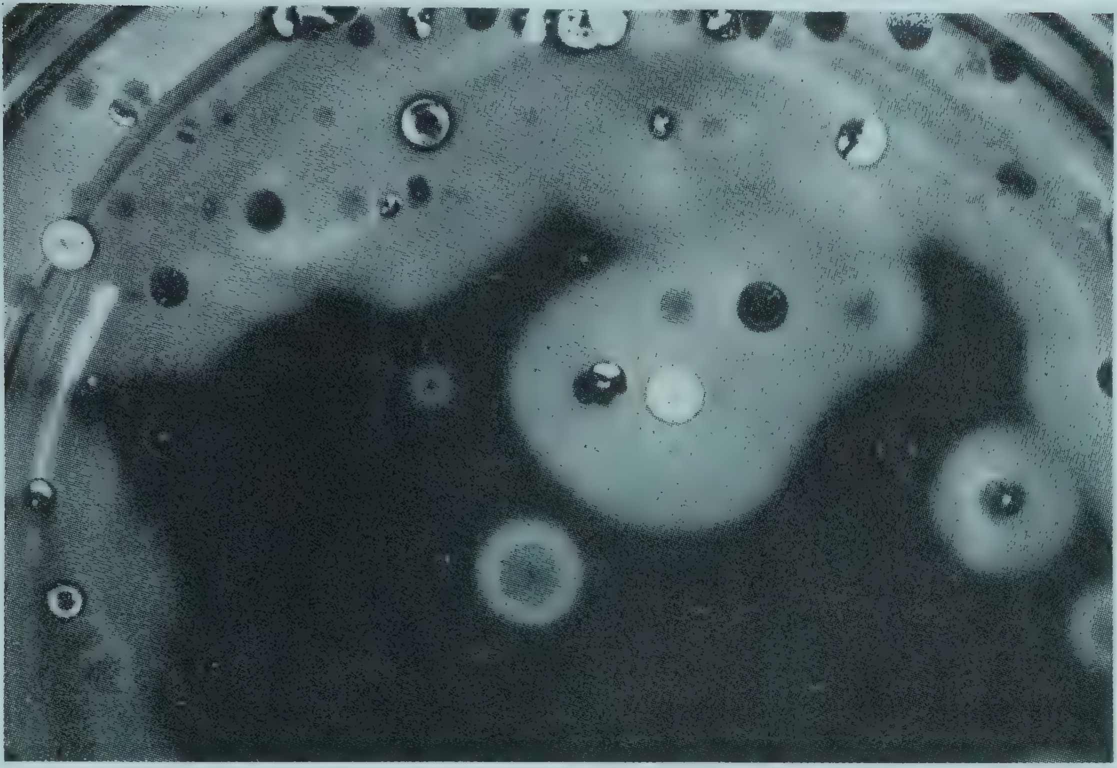


FIGURE 48. Variability of actinomycete colonies in a plate culture (Reproduced from: Stanier, R. Y. J. Bacteriol. **44:** 557, 1942).

amenable to the environment; (b) continuous or fluctuating variations; and (c) developmental variations, resulting in saltations or mutations. The adaptive type is usually characterized by a decrease in the size of the colony, loss of the capacity to form aerial hyphae, reduction in ability to utilize certain nutrients, change in pigment formation, and loss or gain in capacity to produce specific antibiotic substances. The continuous type of variation is marked by the nature and intensity of the pigment formed by the organism, as well as by the capacity to produce a given antibiotic. The developmental variations are also illustrated by the presence or absence of aerial mycelium, pigmentation, and production of antibiotics. Some of these changes can be reversed to the original by growing the organism on special media, such as glycerol nutrient agar or sterile soil.

Other variations or mutations are more nearly permanent or more stable in nature, although they may appear only on rare occasions. Examples of permanent variations are loss of acid-fastness, of pigmentation, and of the ability to form spores.

The actinomycetes are markedly sensitive to their environment: the extent of mycelium formation can be influenced by a change in the composition of the medium. One of Lieske's cultures produced a well-developed, extensively branched mycelium in potato extract; it grew in the form of short, coccus-like chains on nutrient agar; and gave rise to short, sometimes branched, rods in meat extract-peptone bouillon. Waksman found that in the case of *S. reticuli*, the sporogenous hyphae formed verticils on synthetic agar, but showed racemose branching on nutrient agar or on certain inorganic media.

Several forms of hereditary variation among actinomycetes may be listed: (a) transformation of an actinomycete into a mycobacterium-like organism; (b) transformation of an actinomycete into a diphtheroid organism; (c) transformation of anaerobic, short-hyphal-producing forms of actinomycetes into aerobic, long-hyphal forms; (d) change of antibiotic-producing strains into inactive strains that may also be free from aerial mycelium; (e) change of colorless strains into pink variants, accompanied by a change in the nature of the antibiotic-producing capacity; (f) development, among acid-fast organisms which cause infection in animals, of two subtypes, one liquefying gelatin and the other not liquefying gelatin.

Dissociation of pathogenic actinomycetes into aerobic and anaerobic strains has frequently been recorded. Two types of anaerobic colonies have been isolated from the pus of actinomycosis, one smooth and composed of gram-negative rods, and the other adherent and composed of gram-positive filaments; these were looked upon as S and R forms. These variations have often been considered as a part of the life cycle of the organisms.

The causes of variation among bacteria in general and actinomycetes in particular may be briefly summarized as follows:

1. Nature of substrate in which the organism is growing, such as soil *versus* artificial media, solid *versus* liquid media.
2. Nature of the nutrients, including synthetic *versus* organic media, simple *versus* complex media, dilute *versus* concentrated (salt) media.
3. Environmental factors of growth, notably temperature, moisture content, aeration, and reaction.
4. Inoculum, whether vegetative or spore material, whether a heavy mass inoculum or single-cell preparations.
5. Age of culture, whether continuous or frequently transferred.
6. Presence of other organisms that may exert antagonistic or associative effects.
7. Presence of antimicrobial agents, giving rise to the concept of directed variations.
8. Lytic phenomena, including phage effects.
9. Host specificity, in pathogens.

The nature of the variations may be morphological, comprising colony structure and cell structure, or biochemical, comprising formation, both quantitative and qualitative, of important metabolic products

Kriss recognized four types of variation: morphological, cultural, physiological, and applied. These are illustrated by the variability of a culture of *S. coelicolor*, as presented in Table 16.

Duggar *et al.* made a detailed study of the morphological and physiological variability of certain antibiotic-producing organisms belonging to the genus *Streptomyces*. They concluded that these variations proceed in nature as well as in culture, along parallel lines. They were inclined to accept the modern tendency to propose a new name and description for a recently isolated culture rather than go through the existing hazard of "identification," because of the inadequate study of the strains and lack of "comprehensiveness" of published specific descriptions. Although they agreed on the unsoundness of reducing the species concept to "racial or near-biotype rank," they were willing to consider as a basis of species differentiation "minor or single variations of morphological or developmental features, of responses to environmental changes, of differential election of nutrients or of metabolic differences."

On examining 1,298 freshly isolated cultures of streptomyces, Jones found that about 20 per cent showed considerable fluctuation in the production of aerial mycelium, and 6 per cent formed only substrate growth in the first transfer. In a detailed study of the variations of five strains, Jones concluded that although variations were numerous, they were mostly temporary. Saltations (mutations) were the only permanent variations.

Further studies on the variability of antibiotic-producing strains of species of *Streptomyces* have been made by Frommer, Backus

TABLE 16

Comparison of some variable properties of S. coelicolor (Stanier)

Colony size	Pigmentation	Aerial mycelium	Spores	Tendency to autolysis	Stability
Small	Litmus pigment	White, scanty, delayed	Present	Absent	Stable except when transferred in spore stage
Large	Yellow pigment, very little litmus pigment	White, abundant	Present	Absent	Stable
Small	Litmus pigment	Gray, scanty, sectored	Present	Absent	Unstable
Large	Litmus pigment	None	Absent	Absent	Stable
Large	Litmus pigment	White, abundant, often sectored	Present	Absent	Unstable
Small	None	None	Absent	Considerable	Stable
Large	Very small amount of litmus pigment	Scanty, delayed	Absent	Absent	Stable
Large	Maroon pigment, a litmus pigment	Scanty, delayed	Present	Absent	Fairly stable
Very small	None	None	Absent	Considerable	Stable

et al., Shimako, Saito and Ikeda, Ogins, and numerous others.

Morphological Variations

Jensen (1931) obtained from single-cell cultures of *N. polychromogenes* two different variants, one a rod-shaped or R-form, and the other a filamentous or F-form. The R-form produced initially a small unicellular mycelium, which soon divided into bacteria-like bodies that multiplied by cell division, in the manner characteristic of corynebacteria. It gave rise to two subtypes: a soft or "s" and a hard or "h" type. The s-type formed a soft, pasty growth of a red color; the bacteria-like bodies were usually short, blunt, little-branched, and partly acid-fast. The h-type formed a dry, crumbly growth, adhering firmly to the medium and consisting of a longer and more slender cell, less acid-fast than the s-type and with a marked tendency to produce long filaments. The h-type arose spontaneously in, and could also be produced experimentally from, cultures of the s-type. Exposure of the h-type to ultraviolet rays gave rise to a yellow and to a white variety of the s-type. The F-form was considered as a stabilization of the initial mycelial stage of the R-form; it represented an actinomycete consisting of long, delicate, branching hyphae, with a well-developed aerial mycelium, and without any tendency to divide by septa into bacteria-like elements. The F-form arose spontaneously in old cultures of the s-type but not in the h-type; its appearance did not seem to be influenced by external factors.

Wright obtained a similar kind of dissociation in several aerobic species belonging to the genus *Nocardia*. He observed variants that were diphtheroid in nature and formed a soft, highly pigmented growth. These variants were believed to represent a definite phase common to the members of the group and pointing to the close relationship between the actinomycetes and the corynebacteria.

Numerous other observations of a similar

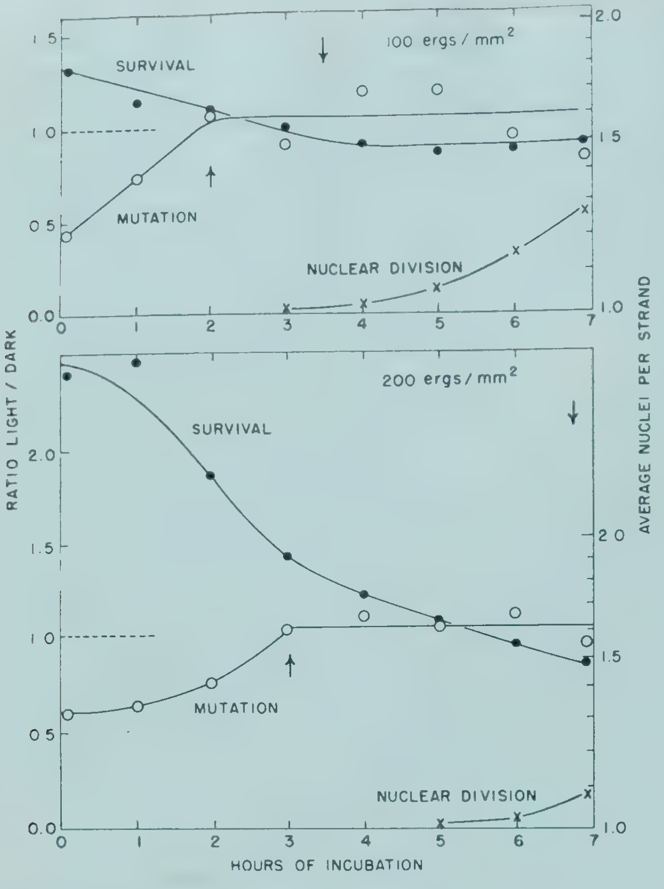


FIGURE 49. Mutagenic and lethal effects of ultraviolet radiation (Reproduced from: Newcombe, H. B. *Mutation*. Brookhaven Symp. in Biol. No. 8, 1956, p. 90).

nature have been reported. It is sufficient to mention the work of Novak and Henrici on the appearance of a yellow staphylococcus in a Berkefeld filtrate of a broth culture of a saprophytic actinomycete. The staphylococcus was found to change first into rods, then into long branched filaments which could not be distinguished from true actinomycete hyphae. The coccus also dissociated first into S- and R-forms, then into filterable G-forms. Krassilnikov described a micrococcus as merely a stage in the normal development of the nocardias, rather than as an abnormal mutant (see also Levy, Karwacki, Koelz).

In general, two opposing concepts served as the basis for explaining the variations among the actinomycetes. On the one hand, there is the two-phase life cycle concept of species, described previously. On the other, there is the earlier and generally accepted complex of the asexual character of the actinomycetes (Jones).

The two-phase life cycle concept of species of *Streptomyces* assumes a haploid substrate mycelium and a diploid aerial growth. According to Badian (1936), chromatin material is distributed through the hyphae in the form of chromosome-like bodies; these unite just before spore formation takes place, so that each spore has a bivalent chromosome. On germination of the spores, the bivalent chromosome undergoes two divisions. One of these is a reduction division; the one to three germ tubes each receive one of the four chromosomes; the remaining chromosomes gradually disintegrate.

Klieneberger-Nobel accepted this concept, designating the fused cell of the primary (substrate, haploid) mycelium as an "initial cell" which produces the aerial or secondary mycelium. Morris also accepted this concept for *A. bovis* and recognized two fusions in its life cycle. According to Webb *et al.*, the nuclear process during fragmentation in *N. corallina* "appears to give rise to binucleated bacillary cells. Coccoidal cells, however, are observed to be uninucleate." The cytological results of Badian were believed by Stanier to provide a possible basis for explaining spore variations. This process of spore formation was thought to be responsible for the great variability of actinomycetes.

Krassilnikov and Tausson suggested other mechanisms of variation operative through a nonconidial phase of growth. According to Schaede and von Plotho, the bodies which Badian took for chromosomes are actually condensed cytoplasm. The long-accepted view is that the streptomyces is an asexual organism in which the individual is differentiated into a substrate mycelium and an aerial mycelium reproductive by means of spores; the latter may arise directly as a branch from any vegetative hypha at any point. This concept has recently been modified through the demonstration of genetic recombination as will be shown later.

Cultural and Physiological Variations

Variations of the pigmentation of actinomycetes are characteristic. They are of particular significance because of the differentiation of many species largely on the basis of the nature and intensity of the formation of pigments on different media. Major subdivisions of the genus *Streptomyces*, especially into sections or species-groups, are also based, most frequently, upon pigmentation.

The key used in Bergey's system of classi-

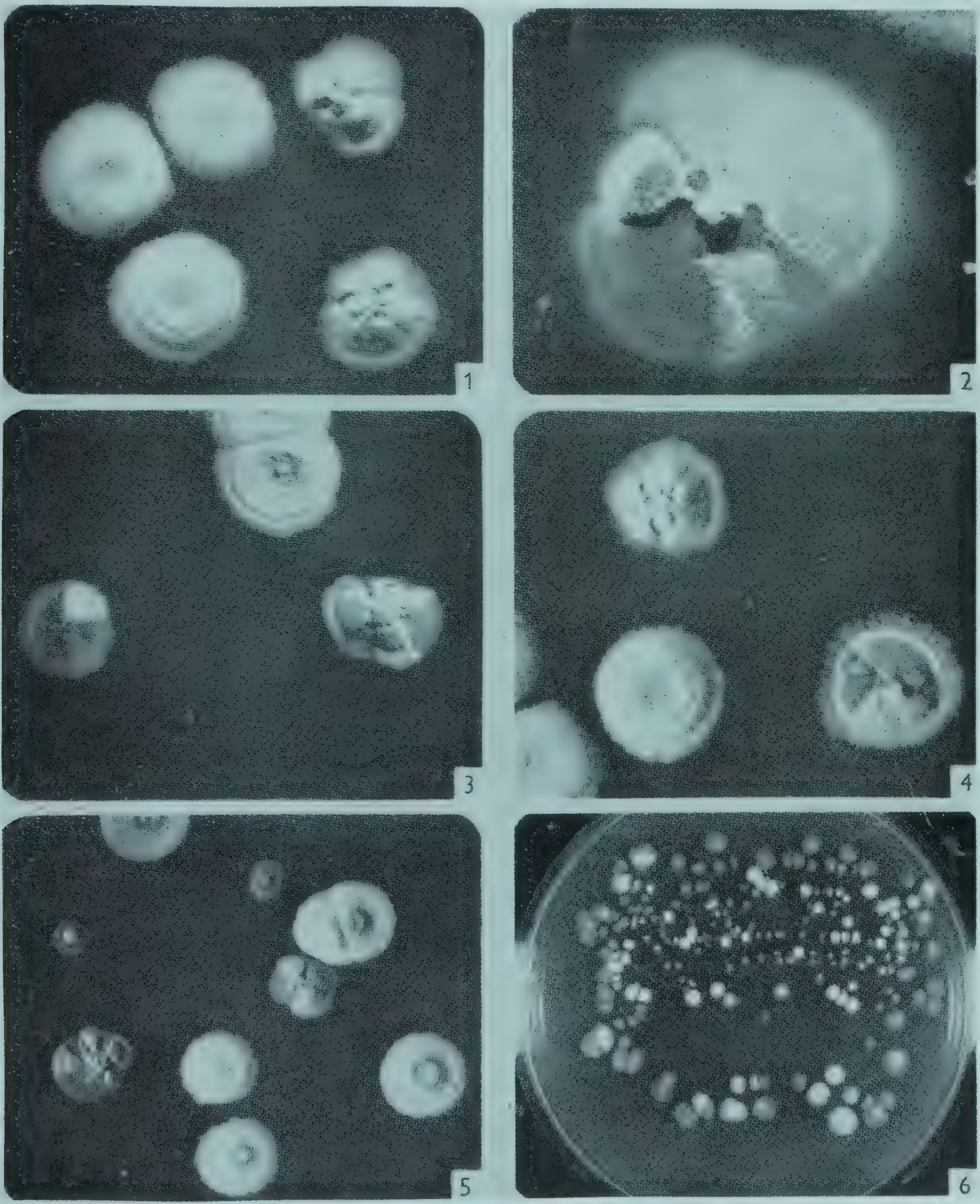


FIGURE 50. Radiation-induced instabilities in streptomyces (Reproduced from: Newcombe, H. B. J. Gen. Microbiol. **9:** 35, 1953).

fication of actinomycetes, especially the genus *Streptomyces* may be taken as an illustration. This key is based largely on the pigments produced on organic and synthetic media. On continued cultivation of the cultures under artificial laboratory conditions, the pigment may undergo changes in nature and intensity, or frequently be lost altogether. When the characters of an organism are based on pigmentation, it becomes very difficult to make comparisons even if type cultures are available. The streptomycin-producing strain of *S. griseus* could hardly be recognized when compared with the original description of the organism made by Waksman and Curtis. The latter was based upon cultures that were at first believed to be similar to one described by Krainsky. Since no one ever had an opportunity to compare freshly isolated cultures with those of Krainsky, the Waksman and Curtis organism is now usually recognized as the type species for *S. griseus*.

S. coelicolor has also been studied extensively, especially from the point of view of pigmentation and agar-decomposition. Erikson observed that the major variations of this organism comprise loss of pigmentation, loss of capacity to produce aerial mycelium, and occasionally loss of ability to liquefy agar. Spontaneous formation of variants could be found more readily in the spores of degenerate colonies, rendered atypical by artificial methods of cultivation. A strain that had lost the power of pigmentation gave a variant which produced sectored colonies, some of which possessed the blue pigment.

Thomas made a study of the variability of *S. scabies*. He isolated six physiologic races that differed in their pathogenicity on 10 different potato varieties. An increase in the nitrogen, phosphorus, and potash content of the medium resulted in a delay in the production of aerial mycelium. Nitrogen and phosphorus were generally favorable for growth, but potash tended to retard it. Maximum growth and stability of the cultures were obtained on peat soil. Mineral soils tended to retard or inhibit growth and increase variability. The more pathogenic races were most stable on most media. These variations led to the question whether the descriptions of many species as causative agents of potato scab represented distinct species or only variants of a single species.

In a study of the variability or mutability of *A. mutabilis*, an organism apparently belonging to the streptomycetes, Masumoto showed that in a synthetic medium, with ammonium chloride as a source of nitrogen, the nature of the carbon source greatly influenced the formation of nonaerial mycelium from aerial mycelium-producing types. Sucrose and lactose were the two sugars most favorable for this purpose. Other carbon sources, notably mannitol, never gave the nonaerial mycelial type.

Attention has already been directed to the marked variations in cultures of *S. griseus* (Schatz and Waksman, 1945; Waksman *et al.*, 1948). Formation of aerial mycelium, pigmentation of the substrate mycelium, production of streptomycin, acid formation, glucose consumption, and autolysis were observed among the qualitative and quantitative variations. The formation of the variants of *S. griseus* were described as follows:

Freshly isolated streptomycin-producing cultures formed typical aerial mycelium, characteristic of the species. These cultures produced an alkaline reaction in glucose-containing media, as well as characteristic surface and submerged types of growth; they underwent only limited lysis and were markedly resistant to the antibiotic action of streptomycin. They gave rise to two kinds of variants:

1. A nonsporulating variant that formed no aerial mycelium and no streptomycin; it was sensitive to the antibiotic action of streptomycin in a glucose-containing medium, and was characterized by a type of

growth that in a shaken culture underwent rapid lysis. This nonsporulating strain could, therefore, hardly be recognizable as typical *S. griseus*; it could almost be considered either as a strain of *Nocardia* or as *Streptomyces sterilis*. Appleby (1947) considered this to be a stable variant, in the nature of mutation, rather than a temporary response to a particular medium or a particular set of conditions. The frequency of this mutant was increased by ultraviolet irradiation of *S. griseus* spores. Greater consideration of the relationship between asporogenous and sporulating types was suggested. Dulaney *et al.* found that some strains obtained from nonsporulating single-colony isolates gave relatively high yields of streptomycin.

2. Another variant produced a red pigmented substrate growth, although there was no visible change in the nature of the aerial mycelium. This variant lost its capacity to form streptomycin but acquired the capacity to produce another antibiotic (rhodomycetin), pigmented red and active only upon gram-positive bacteria. When freshly isolated from the natural substrate, this culture would definitely not be considered as *S. griseus*.

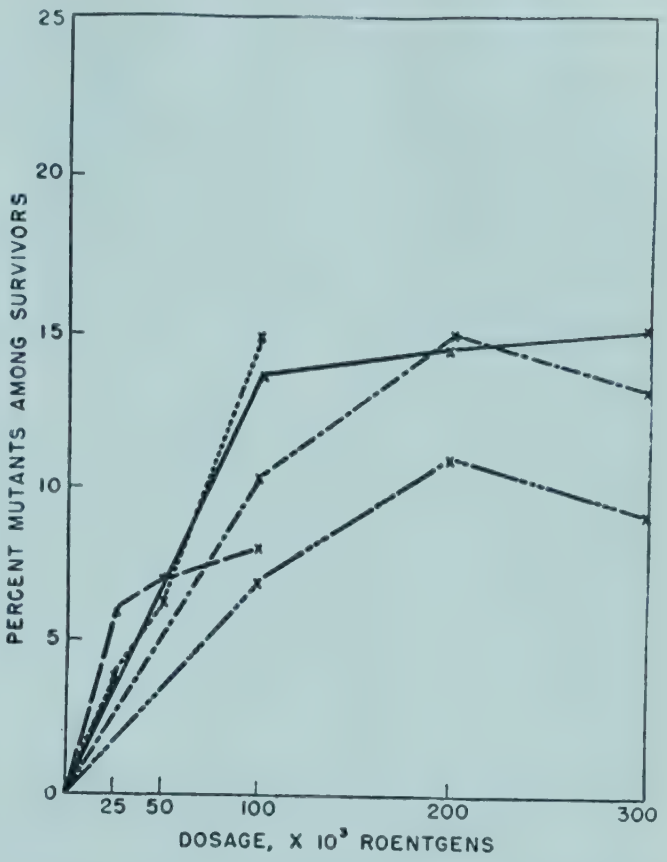


FIGURE 51. Dosage of irradiation and mutation frequency in *S. flaveolus* (Reproduced from: Kelner, A. J. Bacteriol. 56: 463, 1948).

S. lavendulae was also found to yield a number of variants (Waksman and Schatz, 1945; Waksman *et al.*, 1951). These differed in the amount and nature of soluble pigment, in the nature and pigmentation of their aerial mycelium, and in the production of the antibiotic streptothricin. Two variants of this organism were recognized: one gave a bluish colored substrate growth, a blue diffusible pigment, and a lavender-colored aerial mycelium with a slightly blue tinge; the other formed a cream-colored substrate growth, a soluble brown pigment in organic media, and a lavender-colored aerial mycelium. Other variants obtained from this organism gave rise to cultures with white aerial mycelium shaded pink; some were devoid entirely of aerial mycelium, except for a scant growth of sporulating aerial hyphae. These variants also differed in their ability to produce the antibiotic. The conclusion was reached that the formation of streptothricin by *S. lavendulae* is associated with its ability to form aerial mycelium.

Gause and Kochetkova studied the variations of a grisein-producing strain of a streptomyces. Some of the strains formed the pure antibiotic; others formed the antibiotic and a second factor; still others yielded largely the second factor and very little of the given antibiotic. Various transition forms were also obtained.

Krassilnikov (1957) obtained numerous variants or mutants of the antibiotic-producing organisms *S. griseus*, *S. coelicolor*, *S. aureofaciens*, and *S. violaceus* on treatment with various mutagenic agents (radiation, temperature, phages, antibiotics). A study of these variants or mutants revealed there were changes of a cultural and morphological nature; fewer changes of a physiological and biochemical nature; and no change in the antibiotic nature of the organisms. This is quite at variance with the above reports on the production of mutants from the streptomycin-producing *S. griseus* and streptothricin-forming *S. lavendulae*.

Mutations

The concept of "mutation" and the usage of this term has led to a particular controversy. The term has been applied to "sudden changes which are neither the result of a process of gradual acclimatization or education nor of selective isolation." In recent years the subjects of mutations and variations among microorganisms have gained new impetus from studies on the nutrition of the organisms, involving growth factors and metabolites, formation of antibiotics, and development of resistance to a given antibiotic to which they were originally sensitive.

The formation of mutants by actinomycetes has long been recognized. These were considered as special types of variants. The formation of new strains through the mutation of a culture, however, is more fundamental and hereditary. White strains were obtained from blue-pigmented forms; strains free from aerial mycelium, from those producing such mycelium; red strains, from orange-yellow forms. These mutations were accompanied by changes in morphological, cultural, and physiological characters which differentiated the new strains from the mother cultures. The differences thus obtained may be so distinct as to give the new strain a characteristic of a species.

Krassilnikov and his collaborators made a detailed study of such stable mutants. They emphasized that the variations or mutations take place from the simpler to the more complex forms, as from micrococci to mycobacteria, from mycobacteria to nocardias, and from nocardias to streptomyces; the reverse phenomenon occurs but seldom. This reasoning led Krassilnikov to the conclusion that actinomycetes are present in natural substrates, such as soil, largely in the form of micrococci. Kedrovski, however, emphasized that the reverse is true, actinomycetes giving rise to rod-shaped forms of the tuberculosis type.

Spontaneous mutations in stored spores of streptomyces were studied by Wainright.

Saltations

Among the mutations, the phenomena of saltations occupy an important place among the actinomycetes (Rippel and Witter). They are similar in nature to those occurring in colonies of fungi or bacteria. New forms appear in a colony either as sectors or as daughter colonies. These sectors may differ from the mother colony by the presence or absence of aerial mycelium, by a change in color of the substrate or aerial mycelium, by structure or rate of growth of the colony, by presence or absence of "fairy rings," etc. (Lieske, Kriss, Krassilnikov). These saltants, upon careful transfer to fresh media, will produce new stable varieties; they differ from the original species in their morphological, physiological, or cultural properties. Some of these saltants could easily be designated as different species had their origin not been known.

Schaal found as many as nine sectors in a single colony of *S. scabies*. The cultures obtained from these sectors varied in the nature of the mycelium, in the rate of growth, and in the pigmentation. The formation of spirals and the direction of the turns in the spirals were also variable characters. Nutrition exerted a marked effect: production of aerial mycelium was inhibited by a high nitrogen content of the medium; presence of thiamine favored rapid growth and the formation of sectors. There was no correlation, however,

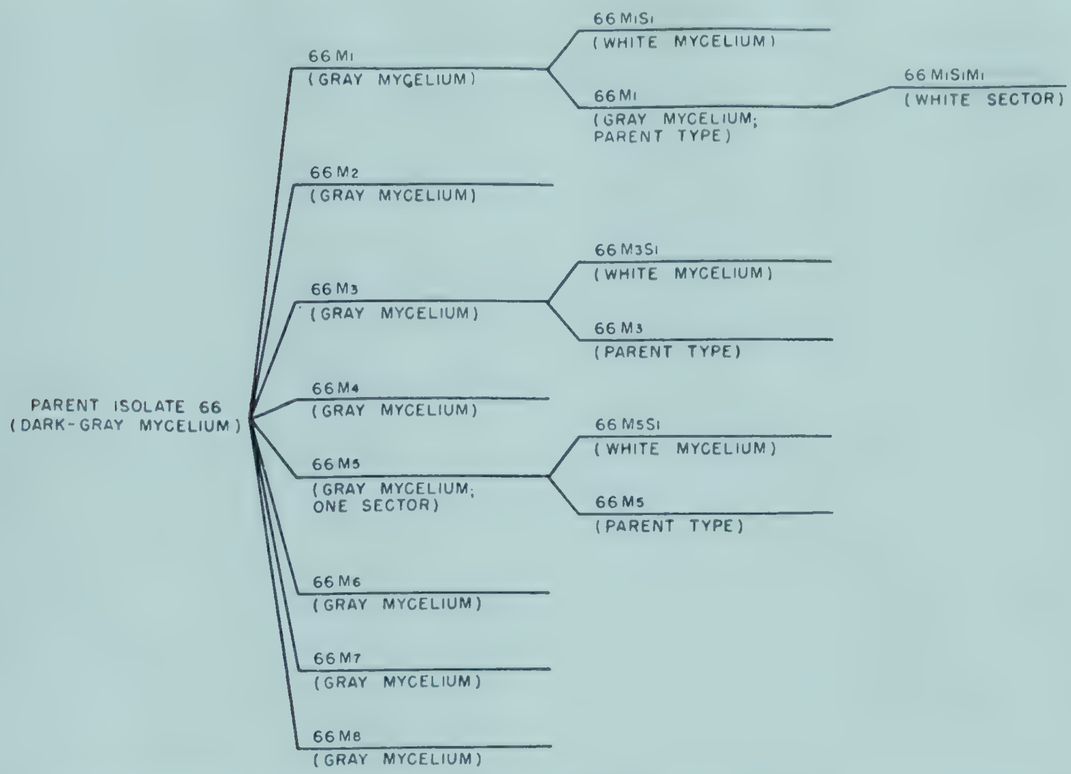

PARENT ISOLATE 66
(DARK-GRAY MYCELIUM)
66 M1
(GRAY MYCELIUM)
66 M1S1
(WHITE MYCELIUM)
66 M1
(GRAY MYCELIUM; PARENT TYPE)
66 M1S1M1
(WHITE SECTOR)
66 M2
(GRAY MYCELIUM)
66 M3
(GRAY MYCELIUM)
66 M3S1
(WHITE MYCELIUM)
66 M3
(PARENT TYPE)
66 M4
(GRAY MYCELIUM)
66 M5
(GRAY MYCELIUM; ONE SECTOR)
66 M5S1
(WHITE MYCELIUM)
66 M5
(PARENT TYPE)
66 M6
(GRAY MYCELIUM)
66 M7
(GRAY MYCELIUM)
66 M8
(GRAY MYCELIUM)

FIGURE 52. Diagrammatic relation of the sectors produced by single-cell cultures of a streptomyces from a parent isolate (Reproduced from: Schaal, L. A. J. Agr. Res. **69:** 173, 1944).

between pathogenicity and cultural characteristics of the strains.

Mutagenic Effects

In recent years, extensive use has been made of the mutagenic effects of irradiation and of certain chemical agents. These have found extensive application in obtaining special strains of antibiotic-producing organisms. It is difficult to state definitely whether we are dealing here with true mutations or with the elimination of certain varieties in a highly variable microbial culture.

Jensen reported that, under the influence of ultraviolet rays, strains of *Nocardia*, isolated from Australian soils, gave rise to new forms; some of these resembled typical species of *Streptomyces* and others were closely related to the mycobacteria. Under the influence of LiCl, mycobacteria gave rise to forms that might be considered as species of *Nocardia*.

Savage reported that ultraviolet rays were less mutagenic, in the treatment of streptomyces, than were x-rays; 0.710 Å and 0.210 Å wave lengths were most efficient. Mutation rates increased with killing rates up to 99.9 per cent of killing. When doses of 1,000,000 roentgens were used, as high as 50 per cent mutation rates were observed on morphological properties and 40 per cent on streptomycin production.

By means of x-ray and ultraviolet light irradiations, Kelner found that many cultures not possessing any antibiotic properties gave rise to antibiotic-producing mutants. A strain of *S. griseus* kept for a long time (more than 30 years) in the culture collection and which was inactive antibiotically was induced to form a mutant that produced streptomycin. The frequency of active mutants ranged from 0.01 to 1.2 per cent; mutants obtained from the same parent culture varied in the nature of the antibiotic they were able to produce or in their antibiotic spectra. The viability of spores exposed to ultraviolet irradiation could be recovered by illumination with visible light, a phenomenon that came to be known as "photoreactivation."

A culture of *S. flaveolus* was treated with x-rays in doses ranging from 25,000 to 300, 000 roentgen units. In one experiment 6 per cent of the spores survived 100,000 r units, and 0.03 per cent 300,000 r units. The survival rate was inversely proportional to the dose. The following mutants were obtained: (a) biochemically deficient strains which grew well on nutrient agar but very poorly or not at all on asparagine glucose agar; (b) strains with pigmentation more intense than or different from that of the wild type; and (c) asporogenous strains. About 24 per cent of the surviving spores in a suspension treated with 200,000 r units were mutants.

One of the procedures for obtaining highly potent antibiotic-producing strains consists in combining ultraviolet or chemical treatment of spores with single-colony isolation. Another method takes advantage of the frequently greater resistance of organisms to the antibiotic they produce, as in the case of streptomycin, in the plating out of cultures in media containing increasing concentrations of the particular antibiotic; the colony that will develop on the plate will tend to represent more potent strains than the original ones. Dulaney *et al.* presented details of the first method, as shown in Figure 46. The most variable characters included color of spores and degree of sporulation; surface and margin of colony, and colony sectoring; amount and color of exudate on colony surface; amount and color of soluble pigment released in substrate. There was also marked variation in amount of streptomycin produced, although no complete correlation could be obtained between the latter and the morphological type. Some of the strains retained the capacity to give high streptomycin yields, and others lost it.

According to Newcombe (Figs. 49 and 50) exposure of spores of streptomyces to ultraviolet and gamma rays results in hereditary changes affecting colony morphology and pigmentation. These changes are largely associated with instabilities that result in further variation during colony growth and spore formation. These instabilities persist indefinitely, giving rise to new variants having their own patterns of instability. These changes differ from gene mutations in that they can be induced with much greater frequency, and that gamma rays are as effective as or more effective than ultraviolet irradiation, suggesting chromosomal rearrangements. Mutations are caused by treatment with x-rays, ultraviolet, and cold, a period of sensitivity during early spore germination being common to all; in the last two treatments, there is dependence on metabolic activity. The phenomenon of photoactivation was studied further by Erokhina and Alikhanyan.

Horváth *et al.* observed that when an antibiotic-producing strain of *S. globisporus* was grown in a sterile filtrate of the macerated mycelium of another antibiotic-producing organism (*S. globosus*), there occurred, after several repeated transfers, a change in the morphological and physiological properties of the first organism. The newly produced strain retained its properties for a considerable time. Cultivating actinomycetes in media containing convallamin changed the staining properties of the organisms and the appearance of the colonies. When grown in ordinary media, such cultures tended to return slowly to the original stage (Hassegawa *et al.*).

According to Krassilnikov, the intrastrain and intraspecies antagonism among antinomycetes is not just a bizarre property but is highly significant in nature and can be utilized for species characterization. This phenomenon has a bearing upon the whole problem of the significance of antibiotics in the part played by actinomycetes in the cycle of nature (Scriabin).

Horváth (1954) suggested that, to raise the productive capacity of cultures of *Streptomyces* of low antibiotic production, the following treatments should be resorted to: (a) ultraviolet irradiation; (b) intensification of vitality by frequent passages; (c) refrigeration. Ultraviolet irradiation increased production of the antibiotic by 40 per cent. Frequent passages gave 50 per cent higher yields. No improvement was achieved by refrigeration. Spore formation on potato blocks was marked in the refrigerated strains; none was observed in the irradiated ones or in those that had undergone frequent passage.

Mashima and Ikeda (1958) made a detailed study of the effects of physical and chemical agents upon induced mutations of *Streptomyces* species. Ultraviolet light, x-rays, and gamma rays effectively increased the reverse mutation of the locus responsible for methionine synthesis. X-rays and gamma rays did not affect the reverse mutation rate at the glutamate locus. A detailed study has been made of the mutagenic activity of 4-nitroquinoline-1-oxide, as shown in Table 17.

Numerous other investigations have been carried out on the variability and mutability of actinomycetes (Temple), especially in connection with their antibiotic-producing properties.

TABLE 17

Mutagenic effect of 4-nitroquinoline-1-oxide on the reverse mutation of glutamate locus (Mashima and Ikeda)

Treatment, hrs	Per cent of survivors on			Mutations per	
	Medium I	Medium II	Medium III	10^7 initial*	10^7 survivors
0	100.0†	70.0	67.0	4.4	4.4
3	79.2	50.5	48.0	4.4	6.2
6	60.0	39.0	35.5	4.6	8.2
9	41.0	22.1	18.6	4.0	12.7
16	10.2	6.0	6.2	2.6	30.4
24	1.9	1.0	1.0	1.8	126.0

* The reverse mutants were counted on medium IV, and the number of colonies at 0 time on medium II, 2.1 × 10^7, was taken as the base of calculation.

† The actual number was 3.0 × 10^{-7}.

Development of Resistance and Problems of Adaptation

Microbial cells vary greatly in their resistance toward their own metabolic products and to various antimicrobial substances. The action of bacteriostatic agents may consist in the prolongation of the growth lag phase, in the reduction of the general rate of growth, or in hastening the rate of death of the bacteria; they may affect one stage or another selectively. When organisms are allowed to grow in the presence of an antimicrobial agent, the concentration of the agent required to bring about a given effect upon the culture is gradually increased. This type of adaptation may be reversed when the cells are again grown in a medium free from the antibacterial agent; sometimes, this type of adaptation may prove to be very persistent.

Adaptation of microorganisms to antimicrobial agents has been explained as follows:

1. Adaptation occurs by natural selection from an initially heterogeneous population. This theory has lost much support since variations have been found to occur in strains derived initially from a single cell.
2. Adaptation occurs by actual modification of the metabolism of individual cells. This may be due to the establishment in the cells of a mechanism alternative to that normally in use, or to the quantitative modification of existing mechanisms.
3. The adaptation is due to a change in some center of organization of the cell.

The mechanism of acquired drug resistance may thus be due either to direct induction or to mutation with selection (Abraham). The ease of development of resistance depends upon the organism and the antimicrobial agent. In some cases the organism may become not only resistant to, but also dependent on, this agent, as was found to be true for bacterial strains requiring streptomycin for their growth.

Variation and the Action of Phage

The lytic properties of actinomycetes, especially under the influence of phage, may also undergo a variety of changes, depending largely upon the development of strains resistant to phage action. When an actinomycete culture is attacked by a phage, the culture will clear up after a few hours as a result of destruction of the sensitive cells. After further incubation, which may sometimes require days, the culture will begin to grow again as a result of development of a variant which is resistant to the action of the phage. This variant can be isolated and freed from the phage and will in many cases retain its resistance to the action of the phage even if subcultured through many generations. Though the sensitive strain adsorbed the phage readily, the resistant variant will generally not show any affinity to it.

The variant may differ from the original strain in morphological or metabolic characteristics, in serological properties, or in colony type. Most often, however, no such correlated changes are apparent, and the variant may be distinguished from the original strain by its resistance to the inciting strain of phage. It has been suggested that the resistance to phage is due to a heritable change of the microbial cell, which occurs independently of the action of the phage. The mechanism may be more complex when the resistant culture does not develop until several days after lysis of the sensitive cells.

The proportion of mutants in a culture and the mutation rate are detected by changes in the colony type produced by the mutant, either in its pigmentation, or in the character of the surface or the edge of the colony. Often, colonies of intermediate character are produced. This is particularly true of cases where the mutation rate is high and where reverse mutation occurs.

Genetic Recombinations

Recent genetic studies on *Streptomyces* have proceeded along two main lines: (a) the radiation genetic studies (Newcombe), for which the uninucleate status of *Streptomyces* spores offers an advantage; (b) studies on genetic interaction among *Streptomyces*. Anastomosis, or the fusion of hyphae, is frequently encountered, with heterokaryosis resulting when both types of parental nuclei persist and multiply in a common cytoplasm. Recombination, based on the exchange of genetic characters between nuclei, is a much rarer phenomenon, reported for *S. coelicolor*, *S. griseus*, and *S. fradiae*.

Sermonti and Spada-Sermonti demonstrated a parasexual process, leading to genetic recombination in *S. coelicolor*. Three types of recombination between strains occurred: (a) strains carrying all the "wild" characters of the original organism; (b) some "wild" characters of one strain and some mutant characters of another strain; (c) mutant characters from both strains.

Bradley and Lederberg used nutritional and resistance markers to establish two parental types of *S. griseus*. They demonstrated that fusion occurs between the hyphae of the two parents, giving rise to heterokaryotic mycelium in which nuclei from both parents were contained in the same hyphae. The spores produced by the heterokaryon were of only one parental type; they were, therefore, considered as homokaryotic. The recombinations did not prove to be stable, however.

According to Bradley, various species of *Streptomyces* can form heterokaryons, *i.e.* associations showing genetic interaction between diverse nuclei in a common cytoplasm. These heterokaryons can be perpetuated by fragments of vegetative mycelium, but not by spores; the latter are uninucleate, being derived from a single nucleus. A strain of *S. coelicolor* forming stable heterokaryons and producing spores with at least two sets of genes was studied. Nutritionally wild type, or prototrophic, colonies were obtained from growth-factor-dependent, or auxotrophic, combinations by two methods: (a) strains were plated together on a minimal medium; the prototrophs arose after 8 to 16 days; (b) strains were grown together on complete medium for 3 to 6 days; the resulting spores were transferred to minimal medium to select prototrophs, which were purified by several serial transfers on complete medium.

Bradley (1958) exposed a population of a single strain of *S. griseus*, for several growth cycles, to a sterile culture filtrate of another strain of *S. griseus*. The first acquired several genetic characteristics of the second strain, namely, streptomycin sensitivity changed to resistance, bacteriophage sensitivity changed to resistance, absence changed to presence of soluble pigment, and presence changed to absence of pigment in the vegetative mycelium. The filtrate contained a low concentration of streptomycin, which did not inhibit the growth of the first strain, but streptomycin-resistant mutants were selected. The observed morphological changes were coupled with bacteriophage and streptomycin susceptibility. The hybridization was said to be the result of selection of mutants rather than gene transfer.

Alikhanian and Mindlin grew biochemical mutants of *S. rimosus* on suitable agar media, and observed that at the point of contact of the mutant colonies more abundant growth and more abundant sporulation occurred. Nuclear fusion and reduction occur in *S. coelicolor* somewhere between hyphal fusion in the substrate mycelium, allowing heterokaryosis and spore production in the aerial hyphae (Hopwood).

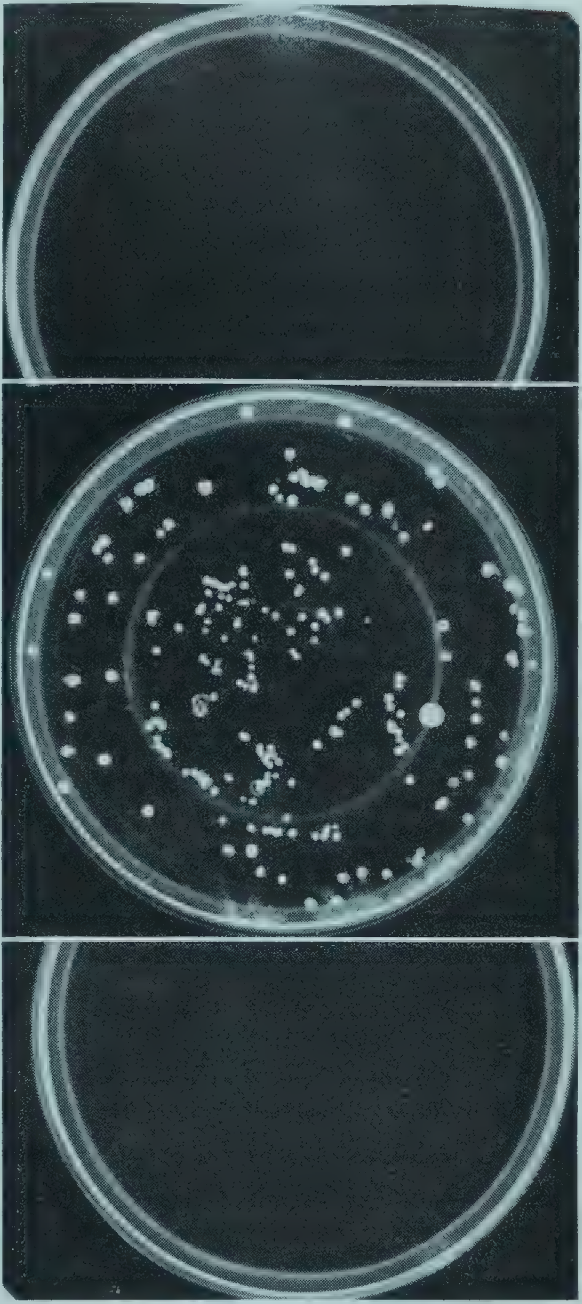


FIGURE 53. Formation of heterokaryotic colonies of *S. fradiae* on minimal agar. Plates 1 and 3 seeded with 10^8 spores of strain 6F4-1 (methionine and isoleucine requiring, and streptomycin sensitive) and 6FS-16 (histidine and arginine requiring, and streptomycin resistant), respectively. Plate 2 received a mixture of parental spores and shows formation of prototrophic colonies (Reproduced from: Braendle, D. H. and Szybalski, W. Proc. Nat. Acad. Sci. 43: 947–955, 1957).

According to Braendle and Szybalski, all of the wild-type strains of *Streptomyces* studied were prototrophic, *i.e.* they formed colonies and sporulated on synthetic agar with ammonium sulfate and glucose as the only nitrogen and carbon sources. Nutritionally deficient mutants were developed by ultraviolet irradiation of the parental prototrophic culture, concentrating the mutants by the filtration technique, and finally employing selective media and the replica-plate principle to detect and identify the mutants. Auxotrophic strains were isolated which required single or multiple supplements of various amino acids or alternative requirements of two, three, and even four amino acids, often as the result of a single mutation. Several antibiotic-resistant mutants were also isolated with the help of the gradient-plate technique (Szybalski, 1958).

The first type of genetic interaction, widely observed in these studies, was the formation of heterokaryotic mycelium containing both types of parental nuclei in a common cytoplasm. This phenomenon was easily demonstrated by plating a mixture of two types of nutritionally marked conidia on a selective medium. A cross was performed between two strains of *S. fradiae*, one streptomycin-resistant and requiring methionine and leucine, and the other requiring histidine and arginine but streptomycin-sensitive (Fig. 53). A mixture, consisting of approximately 10^8 spores from each parent, was incubated for 2 to 6 days on minimal agar. This mixture yielded several hundred prototrophic "recombinant" colonies, whereas the plates seeded with spore suspensions of only one of the parents showed no growth. The formation of heterokaryons, nutritionally balanced, *i.e.* able to grow in the absence of all the nutritional requirements exhibited by any one of the parents, was demonstrated for *S. griseus*, *S. fradiae*, *S. venezuelae*, and *S. albus*. Only *S. coelicolor* produced nutritionally unbalanced heterokaryons, which formed tufts of growth between proximal parental colonies grown on the synthetic medium enriched with a small amount of an amino acid mixture. These unbalanced types did not grow on an unsupplemented medium.

Heterokaryon formation was observed only between mutants derived from the same parental culture. The interspecific crosses and a limited number of intraspecific crosses between different strains designated as *S. griseus* were unsuccessful.

CHAPTER 7

Physiology

Any consideration of the physiology of actinomycetes involves a study of their growth and nutrition, their metabolic processes, and their reaction to environmental conditions. Such important phenomena as saprophytism *versus* parasitism, aerobiosis *versus* anaerobiosis, thermophilic *versus* mesophilic growth, decomposition of organic residues and nitrogen transformation, as well as lytic phenomena and death rate may also be considered here. Some of these processes are sufficiently important to warrant more detailed treatment elsewhere in this volume.

The activites of a microbial cell consist of a multiplicity of chemical reactions, which are interlinked in a most amazing and bewildering fashion. Numerous attempts have been made to base an understanding of the metabolism of the various organisms upon the transformations brought about by resting microbial cells. The capacity of such cells to catalyze the transformation of specific chemical substances has frequently yielded information of considerable biochemical significance. However, the results obtained from such studies have not always been so fruitful in unraveling the complex reactions of microbial cells.

Although our understanding of the physiology of the microbial cell is limited chiefly to a knowledge of the behavior of pure cultures, it is not to be forgotten that, in nature, microbes, especially the actinomycetes, live in constant association with other organisms and are subject to continuous influences of these associated organisms. In the soil and in water basins, each of these microbes lives in association with thousands of others, as well as with the root systems of higher plants and with tissues of higher animals. Some investigators have even asserted that actinomycetes lead only a limited vegetative existence in the soil and occur there largely in the form of spores. The question has, therefore, frequently been raised: How significant are laboratory studies in interpreting the activitives of these microbes in nature? Pathogenic microbes, whether they attack plants or animals, are influenced in their growth and nutrition by the hosts which they inhabit and the tissues which they attack. Physiological reactions based upon pure culture studies and upon the growth of organisms in artificial media may thus be quite distinct from corresponding reactions brought about by the same organisms in a natural environment.

The actinomycetes represent a fairly large group of microorganisms widely distributed in all natural substrates. They represent fairly heterogeneous systems differing greatly in their mode of nutrition, metabolic processes, storage and waste products. Since literally hundreds of antibiotics have been isolated as metabolic products of actinomycetes, one can only surmise the variety of metabolic reactions that led to their formation.

Whenever the chemical composition of a

given organism is discussed, whenever its mode of nutrition and growth characteristics are examined, and its biosynthetic reactions analyzed, it is essential to keep in mind that the conclusions reached hold true for a given environment and for a given set of nutritional conditions. Changing the environment, as by raising or lowering the temperature of growth, by modifying the conditions of aeration, or by changing the reaction, or changing the composition of the medium, as by introducing different nutrients and in different concentrations, will change the growth characteristics and metabolic pattern of the particular organism. Not the least important among these considerations is the recognition of the strain specificity of an organism, whereby certain reactions are limited not to a genus or even a species, but to a certain race or strain.

The metabolism of an organism represents a special phase of its physiology. To comprehend it, we must understand the foodstuffs necessary for the maintenance of its growth and activities; the manner of obtaining the required energy; the products formed as a result of such activities; and the various intermediary reactions through which the nutrients pass when they are used for cell synthesis. Normal metabolism of an organism, when it grows under natural conditions similar to those it finds in a natural environment, is often differentiated from abnormal metabolism, when the growth of an organism is made to deviate from the natural path of life to which it has been accustomed. Such a deviation occurs in virtually all methods used for growing microorganisms on artificial media and under controlled conditions. It is only seldom that a microbe grows in nature in a pure culture. Once it has been isolated and made to grow in an artificial substrate, its metabolism may be considerably modified.

Among the factors influencing the metabolism of microorganisms, the following are most significant:

1. The nature of the energy sources
2. The nature and concentration of the nutrients used for cell synthesis, especially carbon and nitrogen compounds, mineral requirements, and the need for certain rare elements.
3. The need for specific growth-promoting substances or vitamins.
4. The particular oxygen tension of the medium.
5. Optimum temperature and reaction.
6. Influence of other organisms, with the resultant associative and antagonistic effects exerted by them and upon them.

These factors influence the extent of growth of the microbial cell, its chemical composition, and the nature and concentration of specific metabolic products produced.

Metabolism of Actinomycetes

Any comprehensive discussion of the metabolic activities of a group of organisms must consider their utilization of various nutrients, decomposition of these nutrients into simpler compounds, the various mechanisms of transformation of these nutrients, involving those concerned with both breakdown and synthesis, formation of waste products, and a variety of other reactions involved in the life of living cells.

In the very early studies on the growth of actinomycetes, it was found that these organisms vary greatly in their nutrient requirements. Some were found able to consume simple elements and compounds; others required complex organic materials. Considerable adaptation to various nutrients also was observed. The amount of cell material synthesized depended on the availability of the nutrients and on the effect of the accumulated products.

Beijerinck first studied an organism he considered to be a bacillus (*B. oligocarbophilus*), later found to be an actinomycete, that was capable of deriving its carbon and energy needs from some simple compounds present in the atmosphere. Beijerinck and van Delden found that the following elements are essential: N, P, K, and Mg. When a simple synthetic medium to which no carbon compounds had been added was inoculated with a small quantity of soil and incubated at 23 to 25°C, there appeared "a thin, white or feebly rose-colored, very dry film, difficult to moisten." The growth of the film continued for months and resulted in the accumulation of considerable amounts of organic material. Either nitrate or ammonium salt could be used as a source of nitrogen. The carbon was derived from volatile carbon compounds of the atmosphere. Lantzsch, who identified this organism as an actinomycete, differentiated between the nutrition of two variants, one a filamentous form which assimilated CO; the other, a coccus-like or bacillary form, which assimilated aliphatic hydrocarbons. The organism was considered to be an air purifier (Kober).

With the growing recognition of the importance of actinomycetes as producers of antibiotics and vitamins, extensive studies have been made of their metabolic processes.

Waksman, Schatz, and Reilly (1946) found that the growth of *S. griseus* reaches a maximum in stationary cultures in 10 days and in submerged cultures in 3 to 5 days, followed by the lysis of the mycelium. Growth of the organism is accompanied by a gradual rise in the pH value of the culture and in the ammonia and amino nitrogen contents. The total nitrogen in the mycelium tends to be higher during the active stages of growth. The production and accumulation of streptomycin parallels the growth of the organism (Table 18). After maximum activity has been reached, there is a rapid drop, especially in submerged cultures. The production of streptomycin requires the presence in the medium of a complex organic substance, which either serves as the precursor of the streptomycin molecule as a whole or of an important group in the molecule, or functions as a prosthetic group in the mechanism essential for the synthesis of the antibiotic. This substance was designated as "activity factor"; it can be gradually synthesized by the organism. When it is provided in the medium in a preformed state, however, as in meat extract or in corn steep, the process of antibiotic synthesis is greatly facilitated.

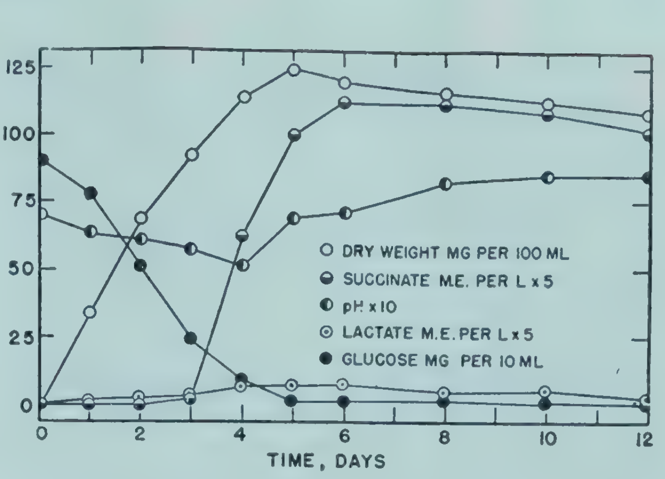


FIGURE 54. Metabolism of *S. coelicolor* (Reproduced from: Cochrane, V. W. and Dimmick, I. J. Bacteriol. 58: 727, 1949).

TABLE 18

Rate of growth and streptomycin production of S. griseus *in stationary cultures* (Waksman, Schatz, and Reilly)

Per 250-ml portion of medium.

Incubation, days	Growth, gm	Nitrogen in mycelium, mg	Streptomycin, μg/ml	NH_3-N in broth, mg	NH_2-N in broth, mg
0				4.3	35.3
4	0.364	35.2	5	22.6	57.3
5	0.437	40.3	8	37.8	69.5
7	0.449	55.8	13	55.9	73.3
10	0.695	62.4	128	63.3	66.8
15	0.640	55.2	140	92.6	79.3
21	0.507	37.6	125	95.1	70.8

In a study of the metabolism of *S. aureofaciens* in complex media containing sucrose and proteins, Biffi *et al.* found that mycelium synthesis is rapid during the first 24 hours then slows down in the next 24 hours. During the first 12 hours, sugar consumption is negligible, while the NH_3 content of

TABLE 19

Metabolic changes characterizing the two phases during the submerged growth of S. griseus (Dulaney and Perlman)

	Phase I	Phase II
Streptomycin	Slight production	Maximum rate of production
pH	Gradual rise	Reaches maximum
Mycelium	Rapid growth	Gradual autolysis
Glucose	Rapid utilization	Small remaining amount exhausted
Soluble carbon	Gradual utilization	Concentration reaches maximum and remains constant
Lactic acid	Slow production and utilization	Slow utilization
Oxygen demand	Maximum	Decreases to minimum
Soluble nitrogen	Used extensively	Concentration increases
Inorganic phosphorus	Used at maximum rate	Released into medium

the culture increases, pointing to the preferential utilization of the proteins as a source of energy. With the advance in growth, sugar utilization and ammonia consumption proceed at a rapid rate, parallel to the increase in dry weight of the organism.

Dulaney and Perlman divided the metabolic processes of actinomycetes into two phases, crescense and senescence. In the first phase, there was uptake of soluble nitrogen, carbon, and phosphate into the mycelium; the oxygen demand was high and the utilization of glucose was rapid, but there was very little antibiotic production. In the second phase, mycelial weight declined, phosphate and nitrogen were excreted into the medium, oxygen demand fell, and streptomycin was produced. Some lactate also formed during the early stages, but this disappeared rapidly (Table 19).

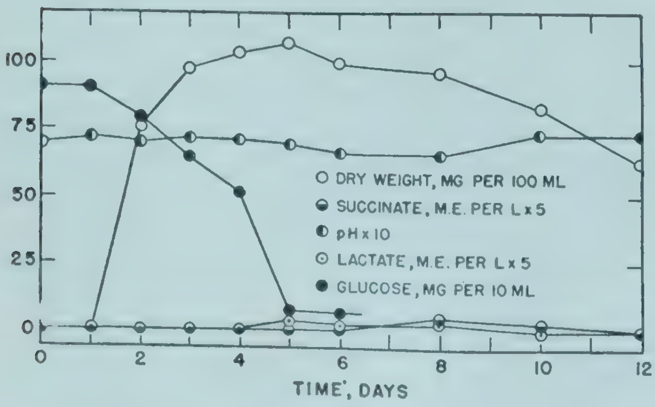
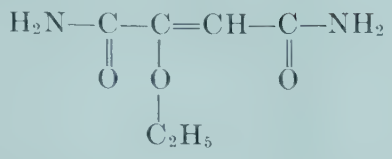


FIGURE 55. Metabolism of *S. griseus* (Reproduced from: Cochrane, V. W. and Dimmick, I. J. Bacteriol. 58: 727, 1949).

Van Dyck and DeSomer also recognized two stages in the growth of a streptomyces (*S. aureofaciens*). The first stage is characterized by cell synthesis and nutrient uptake. The second stage is characterized by a slower increase in cell synthesis; protein and ribonucleic acid decrease, and desoxyribonucleic acid changes but slightly; the decrease in nucleoprotein cannot be ascribed to lysis, since it is not accompanied by a decrease in weight of mycelium or by an increase in the nitrogen in the medium.

The course of metabolism of *S. venezuelae* has been studied by Gottlieb and Legator (1953). The course of growth and the metabolic processes of an actinomycin-producing strain of *S. chrysomallus* were reported by Dietzel *et al.* (1950). Schmidt-Kastner found, for example, that the addition of DL-isoleucine and sarcosine resulted in certain new actinomycins differing in their peptide chains. The metabolic processes of neomycin-producing *S. fradiae* were examined by Giolitti and Lugli (1956).

Numerous other metabolic processes of actinomycetes, notably of members of the genus *Streptomyces*, have been described. Sekizawa (1958), for example, found that a culture of *Streptomyces* produces ethoxyethene-1,2 dicarboamide, as represented by the following formula:



$$H_2N-\underset{\underset{O}{\|}}{C}-\underset{\underset{\underset{C_2H_5}{|}}{\underset{O}{|}}}{C}=CH-\underset{\underset{O}{\|}}{C}-NH_2$$

The nutrition of the various organisms and the effects of certain specific environmental factors may be considered in further detail.

Inoue (1958) reported a high level oxygen demand for *S. griseus* grown on soybean medium at 24 and 96 hours. With deficient aeration and in alkaline media, the secondary high level oxygen demand increased; with an excess aeration the latter disappeared. Streptomycin inhibited the oxygen uptake of young cells at above a certain concentration; this inhibiting action is somewhat prevented by the addition of 10^{-2} *M* Mg and 10^{-3} *M* Mn.

The carbon dioxide output of *S. griseus* gave a maximum rate at 24 hours. The Q_{O_2} value and Q_{CO_2} curve were lower with mycelium than with spore inoculation. The R.Q. value indicated minimum at 48 hours.

Both young and old cells were inhibited by streptomycin, but the inhibitory action was influenced by the relative concentrations of streptomycin and an unknown factor in the medium. The young cells were more sensitive than the old cells. There was believed to exist a difference in the characters (or the enzyme systems) of the young and old cells. The carbon dioxide output was inhibited by streptomycin much more than the oxygen uptake.

Fermentation processes of soybean medium by two strains of *S. griseus* fell into four phases: (a) the amount of mycelium increases, glucose consumption is slight, oxygen demand is high, and streptomycin production is very low; (b) the amount of mycelium is almost constant or slightly increases, glucose consumption is high, streptomycin production increases, and oxygen demand decreases markedly; (c) mycelium increases again and reaches a maximum because of a secondary germination or growth; (d) autolysis occurs, glucose is completely consumed, and streptomycin production reaches the maximum. In the casein medium, the fermentation process falls into 2 phases, and the secondary germination or growth is not observed.

The Q_{O_2} values showed the same tendency both in soybean and casein media. In the latter, the presence or absence of metal salts did not influence the Q_{O_2} value, but it played an important role in streptomycin formation.

Carbon Nutrition

Actinomycetes grow in nature on a wide variety of substrates. The nutrition of actinomycetes can be considered on the basis of the various essential elements required, notably, carbon, nitrogen, and certain minerals, as well as sources of these elements. These sources range from complex organic environments, such as drained peat bogs, high organic soils, and composts of straw or of stable manures, to fairly simple media, such as poor sandy soils and simple synthetic substrates.

Actinomycetes are able to utilize a great variety of organic compounds as sources of energy. These compounds include organic acids, sugars, starches, hemicelluloses and cellulose, proteins, polypeptides and amino acids, nitrogenous bases, and numerous other substances. Some actinomycetes can also attack fats, hydrocarbons, benzene ring compounds, and, to a more limited degree, lignin, tannin, and rubber. There is considerable selectivity in the utilization of these substances by different kinds of actinomycetes. Some of the nutrients, like glucose, maltose, dextrin, starch, glycerol, amino acids, and proteins, are consumed very readily; in fact, they are the best sources of carbon. Sucrose, xylose, raffinose, and certain other sugars, sugar alcohols, and sugar acids are utilized less readily, but more readily by some actinomycetes than by others. Cellulose, chitin, sterols, and polyuronides can be utilized as sources of energy and for cell synthesis by only certain organisms. Each one of these reactions is of considerable biochemical interest. Some of them have been utilized for identification of specific organisms. The biochemical reactions involved are discussed in detail in Chapter 9.

Salzmann (1901) found the following carbon sources most suitable for the growth of actinomycetes (*Streptothrix odorifera*, probably a streptomyces): various carbohydrates and succinic, malic, tartaric, and citric acids. Unsuitable sources were formic, acetic, propionic, butyric, lactic, benzoic, and oxalic acids.

TABLE 20

Carbon utilization by different actinomycetes (Lieske)

Carbon source	*Streptomyces* (No. 12)	*Nocardia* (No. 74)	*Actinomyces* (No. Si)
Glucose	+++*	+++	–
Sucrose	+	+++	–
Maltose	++++	++++	–
Lactose	++++	+++	–
Levulose	++++	+++	–
Dextrin	++++	+++	–
Starch	+	–	–
Inulin	++	+++	–
Glycogen	++	++++	–
Cellulose	–	–	–
Ethyl alcohol	–	++	–
Methyl alcohol	–	++	–
Glycerol	+++	++++	–
Mannitol	+++	++	–
Asparagine	+++	++	–
Tannin	+	+	–
Amygdalin	–	–	–
Caffein	–	–	–
Potassium acetate	–	–	–
Sodium citrate	+	++	–
Blood serum	+++	+++	+
Control	–	–	–

* – = no growth; + = slight; ++ = moderate; +++ = good; ++++ = excellent growth.

Münter (1913) demonstrated that various carbohydrates, organic acids, and alcohols are readily utilized as carbon sources by a variety of aerobic long-hyphal organisms now known to belong to the genus *Streptomyces*.

Lieske (1921) compared the carbon utilization of representatives of three groups: (a) an aerobic, long-mycelial, sporulating form (No. 12), probably a streptomyces; (b) an aerobic, short-mycelial form (No. 74), probably a nocardia, and (c) an anaerobic form isolated from human actinomycosis (Si), probably an actinomyces. One per cent urea was used as a nitrogen source (only the nocardia gave a trace of growth with urea as the only carbon source). The carbon sources were used in 2 per cent concentration. The results are shown in Table 20. Lieske used liquid media in his studies. He fully recognized the fact that had other media and additional cultures been used, the results would no doubt have been different, especially on a quantitative basis. In agar media, for example, starch utilization undoubtedly would have been different.

In general, formic, oxalic, tartaric, benzoic, and hippuric acids are unfavorable carbon sources for actinomycetes; under certain conditions of nutrition, however, some of these can also be utilized by certain organisms. Acetic, lactic, citric, propionic, pyruvic, succinic, and malic are good sources. Ethyl alcohol and ethylene glycol, as well as erythritol and dulcitol, are unfavorable nutrients. Glycerol and mannitol are, on the other hand, highly favorable sources. Starch and certain hemicelluloses, such as mannans, are excellent sources of energy and carbon for a large number of actinomycetes. Utilization of pentose and hexose phosphates has been studied by Cochrane and Hawley.

The significance of carbon utilization, in the form of sugars, organic acids, and alcohols, for diagnostic purposes has been emphasized by Krainsky and Waksman. Arabinose is not assimilated by most species, sucrose is used by some, and cellulose by only a few (Waksman). Inulin is utilized readily by most species.

Gottlieb and Pridham emphasized the selective utilization of some of these compounds in the species characterization of actinomycetes. They found that all species are able to utilize d-glucose, d-mannose, starch, dextrin, and glycerol, but not erythritol, phenol, cresol, and the sodium salts of formic, oxalic, and tartaric acids. Certain compounds are utilized by some organisms and not by others. This is true particularly of rhamnose, raffinose, xylose, lactose, mannose, dulcitol, inositol, and the sodium salts of acetic and succinic acids.

Only certain carbohydrates favor the production of streptomycin by *S. griseus*. These include glucose, starch, and maltose. The addition of inorganic phosphate to *S. griseus* media results in an increased rate of glucose utilization; this is accompanied by almost complete suppression of streptomycin production. Pentoses were found to be poor carbon sources; glucose and mannose were best, especially when combined with proline. Maltose was the best of the disaccharides. The trisaccharides offered inferior nutrients. Inulin was inferior to starch and dextrin. Mannitol was a promising carbon source, but none of the organic acids proved suitable.

Numerof *et al.* reported that, in a medium containing glucose, acetate, and glycine, *S. griseus* utilized only glucose for the synthesis of streptomycin, although the other two compounds also had to be present for efficient production of the antibiotic. All the four carbons of glycine and acetate could account for less than the equivalent of one of the carbon atoms in the streptomycin molecule. More than half of the acetate and glycine carbon appeared as carbon dioxide. The incorporation of labeled carbon from acetate and glycine into streptomycin was thus highly inefficient, although it was still possible to demonstrate localization in the guanidine carbons of the molecule.

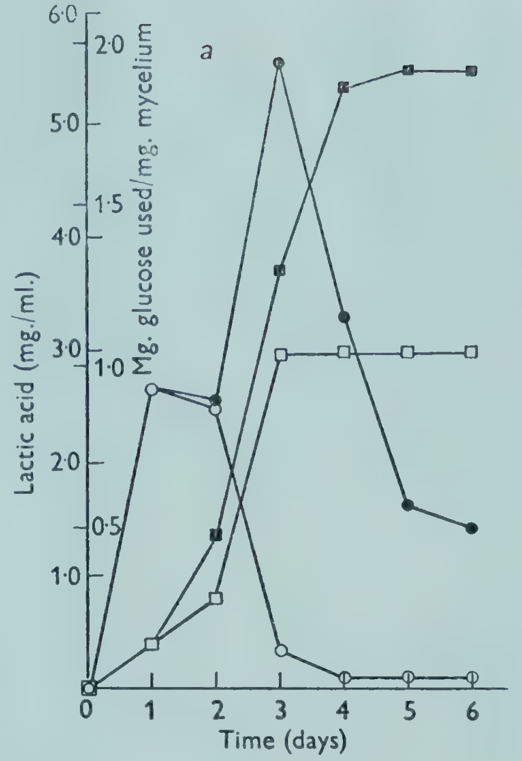


FIGURE 56. Utilization of glucose and production of lactic acid by *S. griseus* (Reproduced from: Hockenhull, D. J. D., *et al.* J. Gen. Microbiol. **10:** 364, 1954).

Benedict *et al.* tested a large number of streptomycete cultures for their ability to utilize various sugars. A total of 147 strains of *Streptomyces* representing 75 species have been tested on 24 carbon compounds for their ability to initiate growth in the synthetic medium of Pridham and Gottlieb. Forty-one cultures were tested also on dulcitol and on the sodium salts of two organic acids. Of the carbohydrates studied L-sorbose was not attacked by any species of *Streptomyces*, and erythritol and dulcitol were found to be of limited value in these tests. Relatively poor growth was attained on melezitose, sorbitol, and esculin (Table 21).

According to Stapp and Spicher, some

TABLE 21

Utilization of various carbohydrates and related compounds by different streptomyces (Benedict *et al.*)

The basal medium of Pridham and Gottlieb was used.

Source of carbon	Number of strains tested	Utilization of carbon source	
		Positive strains	Negative strains
Erythritol	137	9	128
Adonitol	136	46	90
D-Sorbitol	148	31	117
Dulcitol	41	1	40
i-Inositol	147	71	76
D-Mannitol	146	107	39
D-Xylose	133	102	31
L-Arabinose	137	98	39
L-Sorbose	146	0	146
Melibiose	130	48	82
Melezitose	136	45	91
D-Fructose	140	111	29
L-Rhamnose	137	66	71
Trehalose	138	118	20
Maltose	145	141	4
Sucrose	144	38	106
Lactose	142	94	48
Raffinose	143	41	102
Inulin	146	36	110
Salicin	147	106	41
Esculin	145	54	91
Dextran	147	58	89
K-5-ketogluconate	144	32	112
Ca-2-ketogluconate	144	30	114
Na acetate	40	35	5
Na succinate	40	40	0

species of *Streptomyces* are able to grow in high concentrations of carbon sources, such as 80 per cent dextrin, 10 to 20 per cent glycerol, or 20 to 30 per cent glucose.

Kurasawa classified the antibiotic-producing cultures, on the basis of their sugar utilization, into four groups: 1. Rhamnose- and raffinose-negative, 2. Rhamnose- and raffinose-positive, 3. Rhamnose-positive and raffinose-negative, 4. Rhamnose-negative and raffinose-positive. These were further subdivided on the basis of utilization of xylose, lactose, mannitol, and acetate. The streptomycin-producing cultures fell into group 1 and were able to utilize all the four compounds according to the secondary characterization. The streptothricin-producing organisms also fell into group 1, but they were only acetate-positive and xylose-, lactose-, and mannitol-negative. chloramphenicol fell into group 3, and Producers of actinomycins into group 2.

Burkholder *et al.*, found that viomycin-producing strains of *S. floridae* and *S. californicus* utilized xylose, glucose, galactose, fructose, cellobiose, maltose, mannitol, and starch; they grew poorly on arabinose, rhamnose, lactose, sucrose, raffinose, ducitol, i-inositol, and salicin. The grisein-producing strains of *S. griseus*, but not the streptomycin-producing strains, grew well on arabinose and rhamnose (Table 23).

McClung (1954) made a detailed study of the utilization of a large number of carbon compounds by species of *Nocardia*. He found that carbon compounds having an alpha-glucoside linkage (maltose, starch, dextrin, trehalose) are used more often than those having a beta-glucoside linkage (cellulose, lactose). He came to the conclusion that no relationship exists between carbon compound utilization and the morphological groups. Since no two organisms used exactly the same carbon sources, the possibility of using carbon compound utilization as a means of species differentiation was strongly suggested. However, the carbon compounds used by six strains of *N. asteroides* were not the same. This suggested that different isolates of the same organisms differ in their ability to use carbon compounds.

A substance related to vitamin B_1 is effective in stimulating the growth of *N. corallina* (Reader, Peters *et al.*, Lutz). Martin and Batt have shown that this organism requires the addition of thiamine to synthetic media, especially for the utilization of ammonium ions.

Various organic acids, such as malic and

TABLE 22

Classification of antibiotic-producing streptomyces on the basis of sugar utilization (Kurasawa)

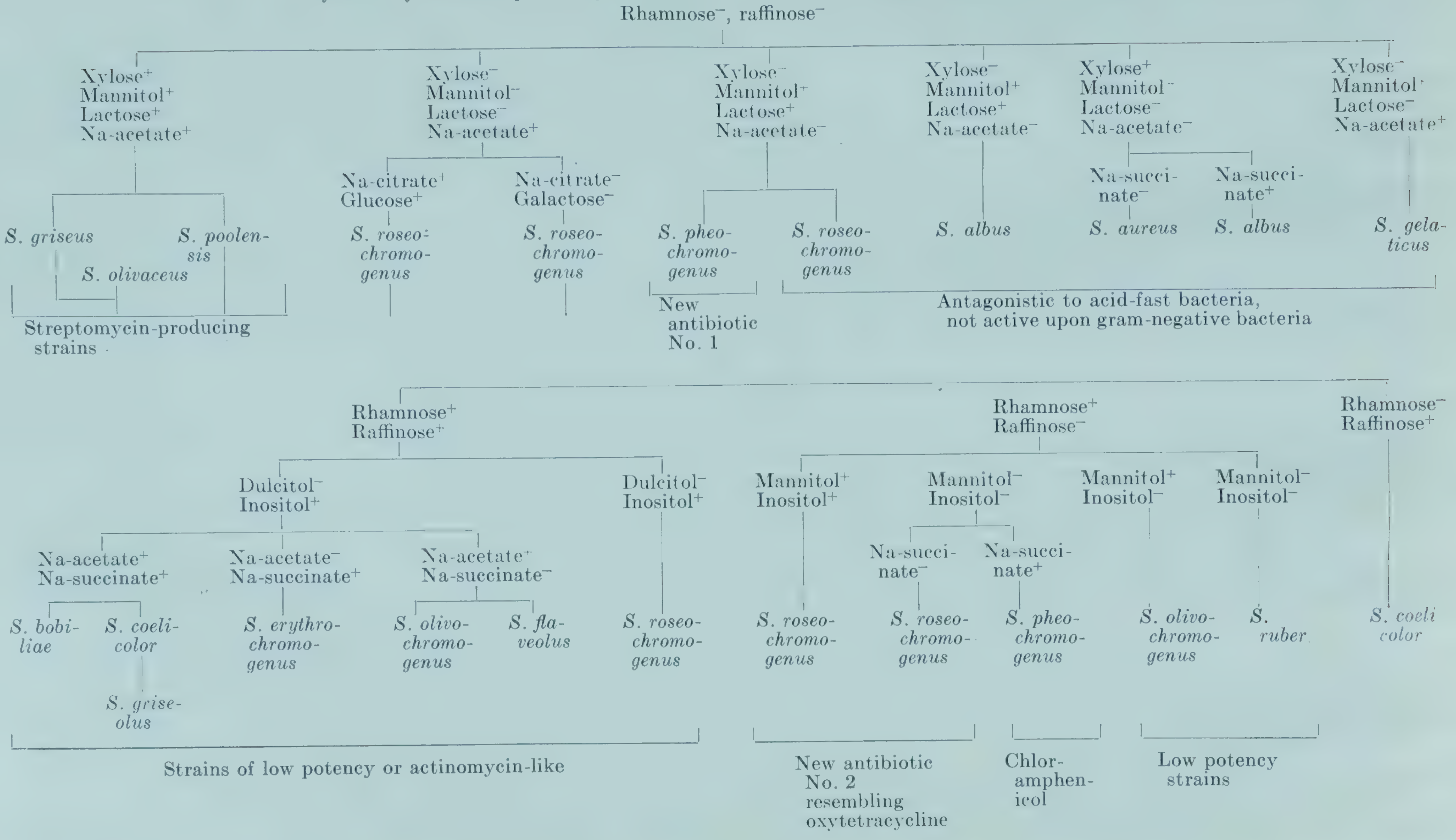

Rhamnose^{-}, raffinose^{-}
Xylose^{+} Mannitol^{+} Lactose^{+} Na-acetate^{+}
S. griseus
S. poolensis
S. olivaceus
Streptomycin-producing strains
Xylose^{-} Mannitol^{-} Lactose^{-} Na-acetate^{+}
Na-citrate^{+} Glucose^{+}
S. roseochromogenus
Na-citrate^{-} Galactose^{-}
S. roseochromogenus
Xylose^{-} Mannitol^{+} Lactose^{+} Na-acetate^{-}
S. pheochromogenus
S. roseochromogenus
New antibiotic No. 1
Xylose^{-} Mannitol^{+} Lactose^{+} Na-acetate^{-}
S. albus
Xylose^{+} Mannitol^{-} Lactose^{-} Na-acetate^{-}
Na-succinate^{-}
S. aureus
Na-succinate^{+}
S. albus
Xylose^{-} Mannitol^{+} Lactose^{-} Na-acetate^{+}
S. gelaticus
Antagonistic to acid-fast bacteria, not active upon gram-negative bacteria
Rhamnose^{+} Raffinose^{+}
Dulcitol^{-} Inositol^{+}
Na-acetate^{+} Na-succinate^{+}
S. bobiliae
S. coelicolor
S. griseolus
Na-acetate^{-} Na-succinate^{+}
S. erythrochromogenus
Na-acetate^{+} Na-succinate^{-}
S. olivochromogenus
S. flaveolus
Dulcitol^{-} Inositol^{+}
S. roseochromogenus
Strains of low potency or actinomycin-like
Rhamnose^{+} Raffinose^{-}
Mannitol^{+} Inositol^{+}
S. roseochromogenus
Mannitol^{-} Inositol^{-}
Na-succinate^{-}
S. roseochromogenus
Na-succinate^{+}
S. pheochromogenus
New antibiotic No. 2 resembling oxytetracycline
Chloramphenicol
Mannitol^{+} Inositol^{-}
S. olivochromogenus
Mannitol^{-} Inositol^{-}
S. ruber
Low potency strains
Rhamnose^{-} Raffinose^{+}
S. coeli color

TABLE 23

Utilization of carbon compounds by viomycin-producing streptomyces (Burkholder *et al.*)

Carbon source	*S. floridae*	*S. californicus*	*S. puniceus*	*S. vinaceus*	*S. griseus* (streptomycin-producer)	*S. griseus* (grisein-producer)
L-Arabinose	0 to ++	± to +	--	0	0 to +	++++
L-Rhamnose	0	0 to ±	0	±	0	++++
D-Lactose	0 to ++	0 to +	0	0	0 to +++	± to +++
D-Maltose	++++	+ to ++++	--	++++	++++	+++ to ++++
Sucrose	0 to ±	0 to ±	0	0	0 to ±	0 to ±
D-Raffinose	0	0	0	±	0 to ±	0 to ±
Inulin	0 to ±	0 to ±	0	±	0 to ±	0 to ±
Starch	++++	+++	++++	++++	+++ to ++++	++++
i-Inositol	0	0 to ±	0	±	0 to ±	0 to ±
D-Mannitol	++++	++ to ++++	++++	++++	+++ to ++++	++++
D-Sorbitol	0 to ±	0 to ±	0	±	0 to ±	±

TABLE 24

The utilization of carbon and nitrogen sources by S. coelicolor (Cochrane and Conn)

Carbon source*	Relative growth†	Relative pigment intensity†	Nitrogen source‡	Concentration, gm/l,	Relative growth§	Relative pigment intensity§
None	17	0	None		30	32
D-Glucose	100	100	L-Asparagine	0.50	100	100
D-Mannose	202	200	Glycine	0.29	83	36
D-Galactose	84	97	L-Leucine	1.00	76	36
D-Fructose	79	96	L-Tryptophan	0.78	98	82
D-Xylose	143	121	Urea	0.24	86	51
L-Sorbose	26	0	$NaNO_3$	0.64	18	0
L-Arabinose	65	46	$(NH_4)_2HPO_4$	0.50	57	0
Starch	107	87	Ammonium acetate	0.58	18	0
Inulin	32	0	Peptone	1.00	146	118
Trehalose	90	38	Tryptone	1.00	91	106
Cellobiose	81	95	Casitone	1.00	116	100
Maltose	62	43	Pepticase	1.00	175	118
Lactose	105	64	Casamino acids	1.00	116	129
Sucrose	34	0	Sodium caseinate	1.00	72	53
Glycerol	135	170	Gelatin	1.00	106	29
Mannitol	82	88	Egg albumin	1.00	44	35
Dulcitol	20	0				
Sorbitol	27	0				
Acetic acid	33	33				
Lactic acid	60	0				
Fumaric acid	69	0				
Succinic acid	47	0				
dl-Malic acid	60	0				
Tartaric acid	20	0				
Citric acid	24	0				
Gluconic acid	82	25				

* Basal medium (gm/l): asparagine, 0.5; yeast extract, 0.5; K_2HPO_4, 0.5; $MgSO_4 \cdot 7H_2O$, 0.25; and minor elements. Carbon source 5 or 10 gm per liter.

† Dry weight and pigment intensity of glucose control taken as 100.

‡ Basal medium (gm/l): glucose, 10.0; yeast extract, 0.5; K_2HPO_4, 0.5; $MgSO_4 \cdot 7H_2O$, 0.25; and minor elements.

§ Dry weight and pigment intensity of asparagine control taken at 100.

citric, are excellent sources of carbon, as shown by Cochrane and Conn (Table 24). In an evaluation of salts of organic acids, Pridham, Hall, and Shekleton (1951) found that the sodium salt of acetic acid appears to be more promising than the sodium salt of succinic acid. They observed that little or no growth was attained in 36 species of *Streptomyces* on Na formate, Na oxalate, or Na tartrate, but that virtually all strains could utilize Na citrate. Nine strains out of 32 produced H_2S in Kligler's peptone-iron agar.

Jagnow reported that even oxalic acid can

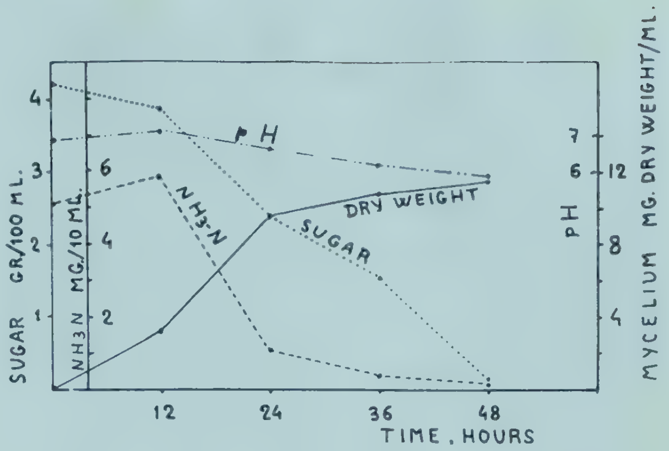


FIGURE 57. Chemical changes during fermentation of *S. aureofaciens* (Reproduced from: Biffi, G. *et al.* Appl. Microbiol. 2: 289, 1954).

TABLE 25

Utilization of carbon sources by viridogrisein- and griseoviridin-producing and related streptomyces (Anderson *et al.*, 1956)

Carbon source	*S. fragilis*	*S. griseoviridis*	*S. griseus*		*S. lavendulae* (streptothricin)
			(grisein)	(streptomycin)	
None	0 to +	0 to +	0 to +	0 to +	0 to +
L-Arabinose	+++ to ++++	+++ to ++++	++++	0 to +	0
L-Rhamnose	++++	+++ to ++++	++++	0	0 to +
D-Xylose	+++ to ++++	++ to +++	+++ to ++++	++++	0 to +
Glucose	++++	+++ to ++++	++++	++++	++++
D-Galactose	++++	+++ to ++++	++ to ++++	++++	++++
D-Levulose	++++	+ to ++	++++	++++	+ to ++
D-Mannose	++++	++ to ++++	++++	++++	++++
D-Cellobiose	++++	+++ to ++++	++++	+++ to ++++	++++
D-Lactose	0 to ++	++ to ++++	0 to +++	0 to ++	0 to +
D-Maltose	++++	++++	++++	++++	++++
Melibiose	0 to +	0	0 to +	0 to +	++++
Sucrose	0 to +	0	0 to +	0 to +	0 to +
Trehalose	+++	++++	++++	++++	+
Melezitose	0 to +	0	0 to +	0	0
D-Raffinose	0 to +	0 to +	0	0	0
Dextrin	++++	++++	++++	++++	++++
Inulin	0 to +	0 to +	0 to +	0 to +	0
Starch	++++	+++ to ++++	++++	++++	++++
Adonitol	0 to +	0	0	+ to ++	0
Dulcitol	0	0	0	0	0
Glycerol	++ to ++++	+++ to ++++	+++	++++	++++
i-Inositol	0 to +	0 to +	0	0	0
D-Mannitol	++++	++++	++++	++++	0
D-Sorbitol	0 to +	0 to +	0 to +	0 to +	0 to +
Aesculin	0 to +	0 to +	0 to +	0 to +	0
Salicin	++ to ++++	0 to +	0 to +	+ to +++	+ to 0 ++++

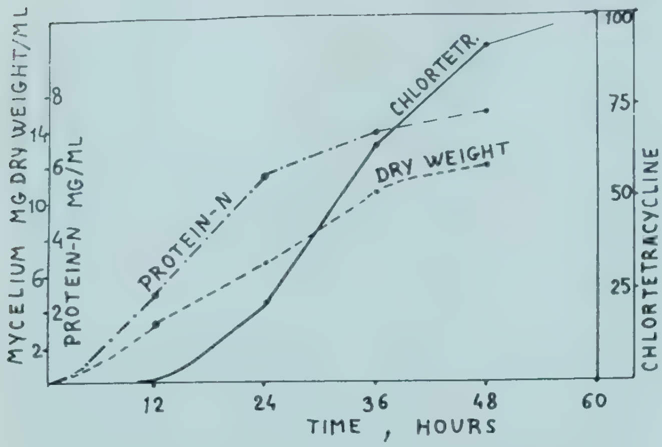


FIGURE 58. Growth and chlortetracycline formation by *S. aureofaciens* (Reproduced from: Biffi, G. *et al.* Appl. Microbiol. 2: 289, 1954).

be broken down by 25 per cent of all actinomycete strains freshly isolated from soil. The presence of yeast extract and the use of ammonium sulfate in place of nitrate as a source of nitrogen greatly facilitate this reaction.

Numerous other studies have been reported on the utilization of various carbohydrates and their derivatives by different actinomycetes, as exemplified in the work of Anderson *et al.* (Table 25).

Mariat (1958) studied the utilization of 15 carbon compounds in synthetic media by various pathogenic strains of nocardia and streptomyces. Glucose was utilized by all strains and fructose by almost all. Paraffin was utilized by all strains of *N. asteroides* and *N. brasiliensis*. Mariat recorded the following series for two species of nocardia and three streptomyces:

N. asteroides: Glucose > fructose > glycerol > mannitol > the other carbon compounds which were practically not utilized.

N. brasiliensis: Glycerol > glucose > fructose > galactose > mannitol > xylose > arabinose > saccharose > maltose > the other compounds which were practically not utilized.

S. madurae: Glucose > glycerol > starch > xylose > mannitol > fructose > saccharose > galactose > maltose > Na acetate > lactose > Na citrate > the other compounds which were not utilized.

S. pelletieri: Glucose > fructose > Na acetate > the other compounds tested which were not utilized.

S. somaliensis: Glucose > maltose > fructose > the other compounds tested which were not utilized.

As sources of energy and carbon, proteins and their derivatives are frequently preferred to carbohydrates by actinomycetes, especially species of *Streptomyces*. This is shown by the fact that when a protein or a peptone is present in the same medium with glucose or another available carbohydrate, an actinomycete may attack the protein first, not only as a source of nitrogen but also as a source of energy and carbon; considerable waste nitrogen is thereby liberated in the form of ammonia. The favorable effect of glucose in increasing the growth of actinomycetes in the presence of protein is due partly to the neutralizing effect on the ammonia produced from the peptone by the acid formed from the glucose. Tyrosine can be used by certain species of *Streptomyces*, with the formation of dark-pigmented compounds. Some of the amino acids, like leucine, are utilized by actinomycetes only in the presence of an available carbohydrate. Urea can serve as a source of nitrogen, but not of carbon.

The metabolic changes involved in the utilization of polypeptides and amino acids as sources of carbon have been studied by Woodruff and Foster (Table 26).

Decomposition of Cellulose, Chitin, and Agar

Among the carbon sources for the nutrition of actinomycetes, cellulose occupies a unique place. The capacity of certain actinomycetes to decompose cellulose is well established. The cellulose-plate method or a liquid medium containing the necessary inorganic salts, a source of nitrogen, and filter paper as a source of cellulose can be used to demonstrate this capacity. Krainsky found that certain pigmented cultures are particularly active in decomposing cellulose. Black or red rings are formed on the paper; on agar plate, clear rings are produced by the colony, indicating cellulose decomposition.

When filter paper is placed in vessels containing a synthetic solution, with ammonium salt or nitrate as a source of nitrogen, and some calcium carbonate, and inoculated with various cultures, many of the cultures will be found growing on the paper above the surface of the medium. When the residual cellulose is determined, a definite ratio will be found to exist between the cellulose decomposed and the nitrogen assimilated.

Meyer isolated a strong cellulose-decomposing culture of an actinomycete that produced a green pigment and an earthy odor. It is difficult to tell from the description whether this organism was a streptomyces or a micromonospora.

Jagnow reported a widespread capacity of soil actinomycetes to utilize chitin, both as a carbon and as a nitrogen source. None was able to utilize keratin, however. Jagnow found that about 50 per cent of all the freshly isolated cultures of streptomycetes, notably members of the *S. albus*, *S. griseus*, *S. diastaticus*, and *S. antibioticus* groups were able to attack chitin. Humm and Sheppard isolated from marine sources three actinomycetes: *S. marinus*, *N. flava*, and *N. atlantica*. Each of them was capable of digesting agar. Both nocardias produced organic acids from carbohydrates more actively than did the streptomyces. The latter utilized organic acids more readily than did either of the nocardias, and therefore its failure to produce an acid reaction in carbohydrate media may be ascribed tentatively to coincidental utilization of any organic acids formed during decomposition of the carbohydrates.

TABLE 26

Metabolic changes and efficiency of carbon utilization by S. lavendulae (Woodruff and Foster)

	Tryptone	Glycine
Mycelium, dry weight, mg	101	106
Glucose consumed, mg	488	782
NH_3-N liberated, mg	4	22
Nitrogen compounds deaminated, mg	92	162
Lactic acid produced, mg	126	58
Volatile acid as acetic, mg	4	13
Conversion of glucose to lactic acid, %	25.8	7.5
Conversion of glycine to acetic acid, %		10.3
Efficiency of carbon utilization, %	24.8	14.3

Utilization of Unusual Carbon Compounds

Many actinomycetes, notably nocardias, show a predilection for unusual types of carbon compounds as sources of energy. This is true of phenols (Gray and Thornton, 1928), pyridine (von Horvath, 1943; Moore, 1949), pyrimidines (Lara, 1952), glycerides (Perlman and Langlykke), and steroids (Turfitt, 1947), chlorine-containing aromatic compounds such as *p*-dichlorbenzene (Erikson, 1941) and chlorohemin (Jensen and Thofern), paraffins (Haag, 1927; Jensen, 1931–1934; Krassilnikov, 1938; Umbreit,

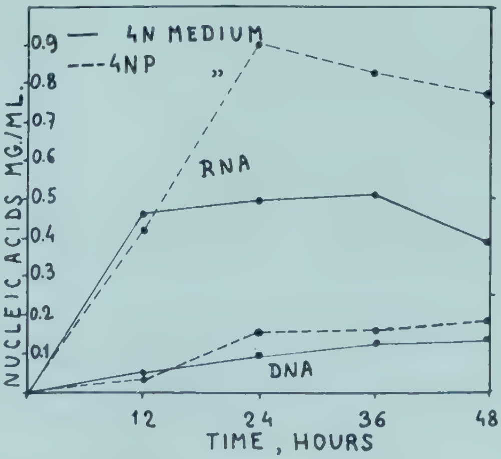


FIGURE 59. Effect of added K_2HPO_4 on nucleic acid synthesis by *S. aureofaciens* (Reproduced from: Biffi, G. *et al.* Appl. Microbiol. 2: 291, 1954).

1939; Erikson, 1949), and other long-chain carbon compounds (Webley and de Kock, 1952). This property is usually associated with oxidative metabolism, potential acid-fastness, production of red or orange pigments of the carotenoid type, and lack of diastatic and proteolytic enzymes. The saprophytic strains, with yellow, greenish, or no pigments (Jensen, 1931–1932; Krassilnikov, 1938; von Plotho, 1948), seem to be devoid of acid-fastness and fail to utilize paraffin, but they are more fermentative and often show diastatic and proteolytic effect.

Nitrogen Nutrition

Proteins, peptones, and certain amino acids form the best sources of nitrogen for actinomycetes, followed by nitrates, ammonium salts, and urea. Actinomycetes are unable to fix nitrogen and have to depend, like the great majority of fungi and bacteria, upon fixed compounds of nitrogen for their cell synthesis.

Münter (1914) made one of the first detailed studies of nitrogen utilization by certain actinomycetes, now recognized as streptomyces. Lieske (1921) used a 2 per cent glucose solution containing a small amount of $MgSO_4$ and K_2HPO_4, and 1 per cent of the various nitrogen sources. Incubation took place at 37°C (Table 27). Lieske recognized that had other species and other conditions of growth been used, different results would no doubt have been obtained.

Table 27

Nitrogen utilization by different actinomycetes
(Lieske)

Nitrogen source	*Streptomyces* (No. 12)	*Nocardia* (No. 74)	*Actinomyces* (No. Si
Potassium nitrate	+	++	–
Ammonium sulfate	+	+	–
Hippuric acid	–	–	–
Uric acid	–	–	–
Urea	++++	+++	–
Caffein	–	–	–
Asparagin	+	–	–
Potassium rhodanate	–	–	–
Peptone	++++	++++	+
Blood serum	+++	+++	–
Control	–	–	–

Table 28

Utilization of different amino acids by a streptomyces as compared to that of a fungus
(Waksman and Lomanitz)

		Growth, dry basis, mg	NH_3-N produced, mg
Glycine	*Trichoderma*	50	24.3
Glycine	*Streptomyces*	59	30.5
Alanine	*Trichoderma*	80	22.0
Alanine	*Streptomyces*	126	39.2
Glutamic acid	*Trichoderma*	218	29.1
Glutamic acid	*Streptomyces*	169	28.4

Fedorov and Iliina (1956) have shown that nitrates and nitrites are excellent forms of nitrogen for various actinomycetes. They are reduced down to ammonia and assimilated for all syntheses. The reduced forms of nitrogen (ammonia and hydroxylamine) are also readily utilized, but in lower concentrations. Organic nitrogen sources (urea, amino acids, peptone) are utilized even more readily. Cell synthesis, however, is low, not exceeding 10 to 12 per cent, when the ratio of C:N is less than 20:1 in the organic substrate, free ammonia accumulates. The nitrogen content of the mycelium varies with the ratio of carbon to nitrogen in the substrate.

The great majority of actinomycetes belonging to the genus *Streptomyces* are able to liquefy gelatin and utilize casein. The nocardias, as a rule, are unable to do so. Many of the actinomycetes are able to coagulate and later peptonize milk, though peptonization frequently occurs without previous coagulation. Blood serum is liquefied by many streptomyces. Complex proteins, such as hoof meal and horn meal, can also be attacked by certain forms, such as *S. fradiae*.

A study of the comparative utilization of different amino acids by a streptomyces and a fungus has been made by Waksman and Lomanitz, as shown in Table 28. In some cases, the actinomycetes are even more efficient than the fungi. The comparative decomposition of plant proteins by a streptomyces and a fungus is shown in Table 29.

The utilization of nitrogen sources by *S. griseus*, from the point of view of streptomycin production, has received considerable attention (Dulaney, 1948).

The range of utilization of various nitrogen compounds by certain nocardias and streptomyces grown in synthetic media were reported by Mariat (1958) as follows:

N. asteroides: Asparagine > urea > casein hydrolyzate > $PO_4H(NH_4)_2$ > NO_3K > NO_3NH_4 > $SO_4(NH_4)_2$ > NO_2Na; the last compound was not utilized.

N. brasiliensis: Casein hydrolyzate > $PO_4H(NH_4)_2$ > NO_3K > asparagine > urea > $SO_4(NH_4)_2$ > NO_3NH_4 > NO_2Na which was not utilized.

S. madurae: $PO_4H(NH_4)_2$ > urea > asparagine > casein hydrolyzate > NO_3K > NO_3NH_4 > $SO_4(NH_4)_2$ > NO_2Na which was not utilized.

S. pelletieri: Urea = asparagine = casein hydrolyzate = $PO_4H(NH_4)_2$. The other compounds were not utilized.

S. somaliensis: Casein hydrolyzate > asparagine. The other compounds were not utilized.

Yagashita and Umezawa (1951) studied the nitrogen utilization of *S. phaeochromogenes*, an organism that produces chloramphenicol in natural media and in synthetic media containing glycerol, sodium nitrate, and different amino acids. They observed that alpha-aminobutyric acid, norvaline, leucine, phenylalanine, thyroxine, methionine, lysine, and tryptophan increased the production of the antibiotic over that given by the basal medium; while glycine, alanine, valine, isoleucine, serine, glutamic acid, cystine, and histidine had little effect and in some instances gave less than the basal medium. The most effective was phenylalanine. Alpha-aminobutyric acid, methionine, and serine, if added to a medium containing phenylalanine, increased the production of antibiotic; norvaline, leucine, lysine did not have this effect.

Table 29

Decomposition of plant proteins by different microorganisms (Waksman and Starkey)

Protein	Glucose	Organism	Protein decomposed, mg	Cell growth, mg
Edestin	−	*Trichoderma*	541	167
	+	*Trichoderma*	375	359
	−	*Streptomyces*	162	46
	+	*Streptomyces*	348	187
Gliadin	−	*Trichoderma*	854	151
	+	*Trichoderma*	495	460
	−	*Streptomyces*	240	78

Corum *et al.* (1954) showed that *S. erythreus* produced an antibiotic in synthetic media containing glycine and that the microorganism synthesized alanine first, then valine, and later several amino acids and small peptides appeared.

Sackmann (1956), studying a streptomyces related to *S. roseochromogenes*, which produces an antibiotic on synthetic media composed of a basal medium containing several amino acids, asparagine, and urea, showed that aspartic acid, glutamic acid, glycine, alanine, asparagine, and urea gave good production, while gamma-amino-butyric acid, leucine, isoleucine, methionine, and cystine gave very poor production of the antibiotic.

Dulmage tested the utilization of 23 amino acids and other nitrogenous compounds by *S. fradiae* for growth and neomycin production. Best growth was obtained with gelatin and a casein digest, and with L-arginine, D-glutamic acid, L-glutamic acid, L-histidine, L-lysine, L-proline, and

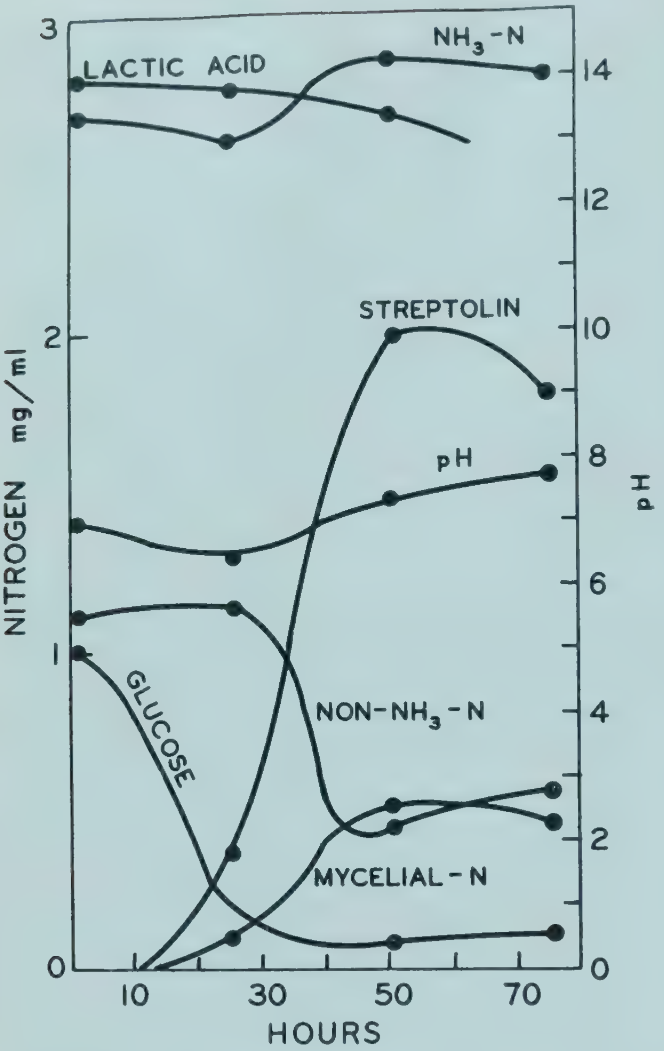


FIGURE 60. Metabolic changes produced by a streptolin-forming streptomyces. Streptolin, units 10^3 ml; glucose, mg/ml; lactic acid, mg/ml (Reproduced by special permission from: Rivett, R. W. and Peterson, W. H. J. Am. Chem. Soc. **69**: 3007, 1947).

DL-threonine; best production of neomycin, with alpha-alanine, L- and DL-aspartic acids, L- and D-glutamic acids, L-histidine, L-proline, DL-threonine, and N-Z amine.

Some actinomycetes, notably nocardias, attack proteins to a rather limited degree. Casein may not be hydrolyzed. Even gelatin, which is readily used by the great majority of streptomyces, is attacked by only some nocardias, and frequently not very readily. In general, nocardias are unable to utilize xanthine, tyrosine, and certain other amino acids. The presence of glucose does not have the same depressing effect upon the decomposition of amino acids by actinomycetes, as shown by the amount of ammonia liberated, as it does upon fungi (Waksman and Lomanitz).

A study has been made of the ratio of carbon to nitrogen consumption by *S. lavendulae*. This was found to depend upon conditions of growth, nature of organism, and age of culture. With sugar and tryptone in the medium, the ratios increased to about 300 per cent as growth advanced, resulting in greater oxidation of the carbohydrate as compared to the utilization of the nitrogen in tryptone for cell synthesis. This was true especially for submerged cultures: the abundance of available oxygen brought about a greater oxidation of carbohydrate as compared to the tryptone consumed (Woodruff and Foster).

Romano and Nickerson (1958) studied the utilization of amino acids as sole sources of carbon and nitrogen by *S. fradiae*. Alanine, histidine, lysine, glutamic acid, proline, and arginine supported growth; aspartic acid, threonine, leucine, isoleucine, and methionine did not. A coenzyme I-linked glutamic dehydrogenase was found in a cell free extract of the organism. It was suggested that this was the mechanism by which members of the glutamic acid series are utilized via the tricarboxylic acid cycle.

Certain actinomycetes, when growing in a peptone medium without any carbon sources, are able to produce urea (Guittonneau). Out of 477 cultures of streptomyces isolated by Stapp, 177 were able to use urea readily as a source of nitrogen. Some of the cultures used as sources of nitrogen, xanthine, hypoxanthine, and adenine, in concentrations of 0.05 to 0.1 per cent. A large number also used uric acid, but not uracil, pyridine, imidazol, and pyrrhol.

According to Moore (1949), certain species of *Nocardia* are able to utilize pyridine, aniline, nicotinic acid, and nitrobenzene as a source of nitrogen, and phenol plus ammonium ion as the sole source of carbon, nitrogen, and energy.

Changes in reaction as a result of growth of various actinomycetes depend both on the organism and on the composition of the medium (Waksman and Joffe). Protein-rich media give an alkaline reaction even in the presence of sugars, largely because of ammonia accumulation. When actinomycetes are grown on media containing sugars and ammonium sulfate as a source of nitrogen, the media usually turn acid as a result of the consumption of the ammonia and the accumulation of sulfate. Media containing sugars and sodium nitrate may first turn acid, then alkaline, as a result of the consumption of the nitrate and the accumulation of the sodium ion in the medium. The great majority of actinomycetes prefer a neutral or a slightly alkaline reaction for their growth. Very few prefer an acid reaction.

Oxygen Consumption by Actinomycetes

The oxygen uptake by *S. lavendulae*, with glucose and glycerol as sources of carbon, was found to be 60 and 45 per cent, respectively. The incomplete oxidation was due to assimilation of some of the products for cell synthesis and to the formation of incompletely oxidized products, such as lactic acid.

Shaking and motion of cultures of actinomycetes usually do not affect all forms alike. The effect of motion has been compared to that of temperature: in both cases, energy is brought from the outside to the living cells; this exerts a favorable action up to a certain limit; above that limit, the results may become unfavorable. Shaking the culture brings about an exchange in the atmospheric gases, the organism obtaining a continuously fresh supply of oxygen. This affects favorably its growth and activities.

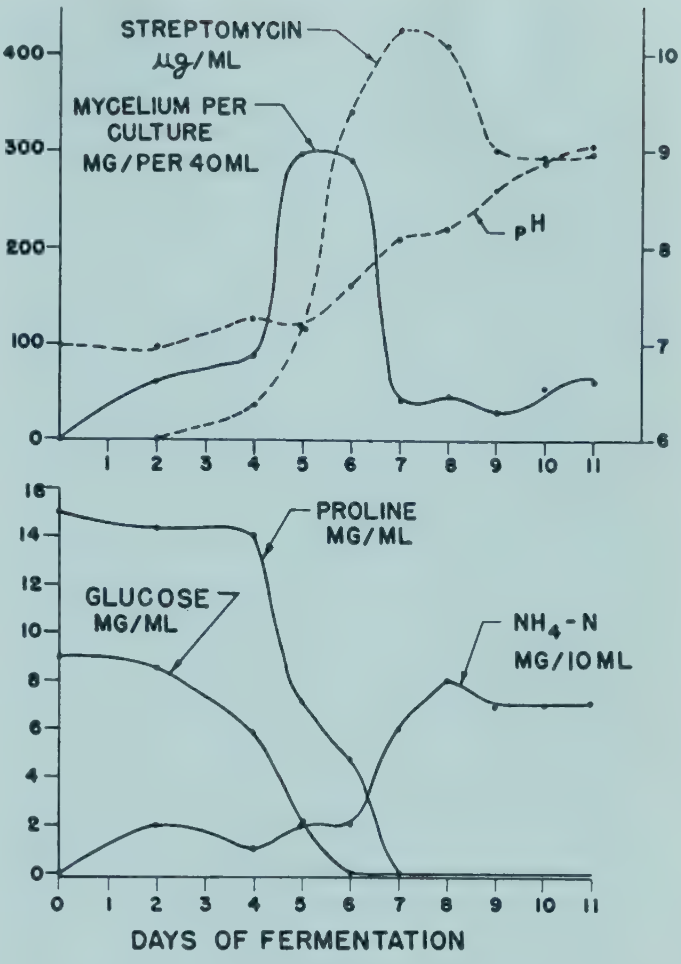


Figure 61. Fermentation characteristics of *S. griseus* (Reproduced from: Woodruff, H. B. and Ruger, M. J. Bacteriol. 56: 316, 1948).

The type of growth produced by an actinomycete in submerged culture varies greatly from stationary growth. In place of the ordinary mycelial mat of stationary growth, the growth in submerged culture is limited to flakes or to bead-like masses or pellets which may fill the whole container. The physiology of the organism may also be markedly affected, one type of antibiotic being produced, for example, in stationary cultures and another type in submerged.

Pine and Howell studied 11 strains of the *A. israeli* and *A. naeslundii* types. These strains were found to require CO_2 for anaerobic growth. Some of them were found to be obligate anaerobes to microaerophiles, others were facultative anaerobes. Further studies of the respiration of *Streptomyces* species have been carried out by Cochrane *et al.*, Kemp and Sayles and many others.

Autotrophy among Actinomycetes

As pointed out previously (Chapter 3), Beijerinck and van Delden in 1903 isolated from the soil a nonmotile coccus and a rod-shaped organism that grew in pure mineral solution and produced, after 2 or 3 weeks, a snow-white, nonwettable surface pellicle. They suggested that the cultures grew at the expense of the traces of volatile organic compounds found in the laboratory air. Later, Beijerinck (1913, 1914) reported that the cultures could also utilize H_2 in the atmosphere. The two cultures were described as *Actinobacillus* (*Bacillus*) *oligocarbophilus* and as *Actinobacillus* (=Streptothrix Cohn) *paulotrophus*, a thread-forming organism. In 1922, Lantzsch cultivated, from a surface pellicle produced spontaneously on a quartz suspension in water, a culture similar to Beijerinck's *Bac. oligocarbophilus* and found it to be closely related to the actinomycetes. He designated it as *Actinomyces oligocarbophilus*. CO was used as a source of C.

Kober (1929) obtained, from an enriched culture of algae heated for 5 minutes at 71° C, a pure culture of a white actinomycete which was related to the cultures of Beijerinck and Lantzsch. Krassilnikov (1938) placed the culture among the proactinomycetes (*Nocardia*).

Ware and Painter (1955) isolated from clear sewage, on a mineral medium with KCN as the only C, N, and energy source, a "strongly autotrophic" actinomycete.

Takamiya and Tubaki (1956) observed an actinomycete growing on a phosphate solution. The culture could be grown in a pure mineral solution. The culture grew chemo-autotrophically, utilizing the process of H_2 oxidation in order to assimilate CO_2. It was named *S. autotrophicus*. Three other cultures of chemo-autotrophic actinomycetes were isolated. Hirsch demonstrated that a culture of *Nocardia*, *N. petroleophila*, grew slowly but steadily upon a purely mineral medium in contact with laboratory air. No growth took place without CO_2. By means of tracer technique, it was established that the CO_2 is assimilated and incorporated in the cell substance. As energy sources for the CO_2 assimilation, the organism uses volatile, aliphatic hydrocarbons with 9 to 14 carbon atoms. These are incompletely oxidized, with the uptake of oxygen and release of only small amounts of CO_2.

Metabolism of Aerobic Actinomycetes

Garner *et al.* (1950) have shown that high-streptomycin-yielding strains had a lower respiratory activity (slower use of glucose

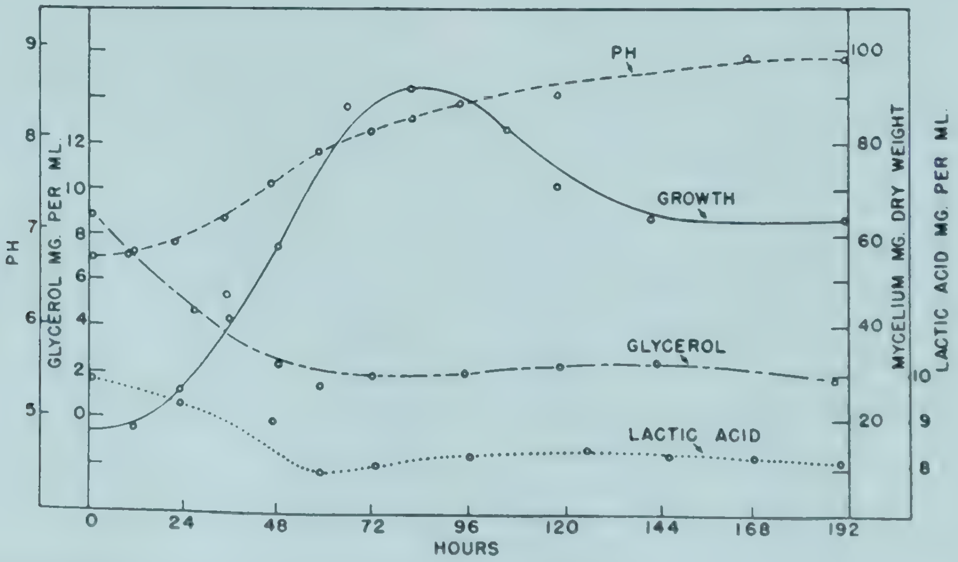


FIGURE 62. Utilization of glycerol and sodium lactate by *S. venezuelae* (Reproduced from: Gottlieb, D. and Legator, M. Mycologia 45: 512, 1953).

and slower CO_2 evolution) and a higher cell dry weight in the initial stage of growth than did the low-yielding strains. High-yielding strains showed active production of volatile nitrogen compounds during the first day, followed by a decrease during the next few days when streptomycin production was at its highest, and then by a slow reappearance of the nitrogen compounds during autolysis. In low-streptomycin-yielding strains, release of nitrogen compounds during autolysis was more pronounced. Added phosphate reduced streptomycin yields. Added calcium increased the yield, probably because of the formation of insoluble phosphates.

Typical fermentation diagrams were prepared by Perlman and Wagman. The pH curve for fermentations in glucose-containing media was typical not only of *S. griseus*, but also of most species of *Streptomyces*. Addition of extra phosphate to the medium depressed streptomycin production and at the same time increased the rate of sugar consumption.

Biffi *et al.* found that addition of small amounts of phosphate to the medium results in increasing consumption by the streptomyces of sucrose and the accumulation of pyruvic acid. In the first stage of growth there is an increase in desoxyribonucleic acid synthesis. There is also a delay in protein decrease on addition of phosphate; this is accompanied by a lower antibiotic (chlortetracycline) yield.

Cochrane and Peck (1952) have shown that whole cells of *S. coelicolor* oxidized some compounds of the tricarboxylic acid cycle. The cells failed, however, to metabolize citrate and α-ketoglutarate. Cell-free preparations oxidized glucose (in presence of adenosine triphosphate), citrate, α-ketoglutarate, succinate, fumarate, and malate; they also decarboxylated oxalacetate. Selected reactions or groups of reactions found to be catalyzed by cell-free extracts included the oxidation of citrate to α-ketoglutarate, the conversion of malate to pyruvate, and the condensation of malate and acetate (or pyruvate) to citrate. The effects of diphosphopyridine nucleotide on malate and fumarate oxidation and of malonate on the oxidation of α-ketoglutarate were consistent with the operation of a tricarboxylic acid cycle.

Metabolism of Anaerobic Actinomycetes

Anaerobic actinomycetes show only relatively limited growth and biochemical ac-

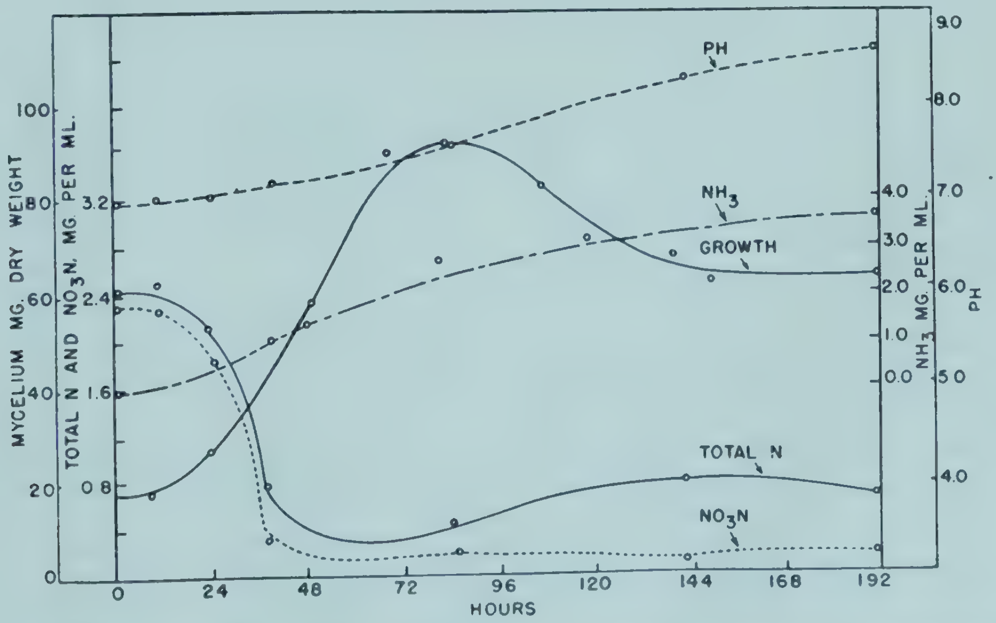


FIGURE 63. Changes in nitrogen-components of medium during growth of *S. venezuelae* (Reproduced from: Gottlieb, D. and Legator, M. Mycologia 45: 512, 1953).

tivity. According to Erikson, they do not attack egg or blood serum; they do not clot or hydrolyze milk; they seldom grow on gelatin; they have little or no hemolytic action on blood agar. Certain strains isolated from human infections have been found to show a slight degree of hemolysis on blood-agar plates at different times, but not consistently. They do not produce soluble pigments on protein media or insoluble pigments in their cells.

The growth of *A. bovis* on sugars is not accompanied by gas formation. Glucose is the most readily available source of energy; acid is formed. Maltose, lactose, and sucrose are also utilized by all strains. Positive or negative reactions with salicin and mannitol have been found of value in differentiating strains, such as human *versus* bovine. *A. bovis* was found by Rosebury to have a limited tolerance for oxygen, which varies, however, among strains.

The introduction of synthetic media for the growth of actinomyces (Howell and Pine) made possible the study of the ability of these organisms to produce lactic acid. According to Erikson and Porteous (1953), *A. israeli* has the capacity to convert as much as 30 to 60 per cent of the glucose utilized to lactic acid, under suitable conditions of growth.

Like aerobic actinomycetes, *A. bovis* is killed by heating at 62 to 64° C for 3 to 10 minutes, but it apparently survives drying for a long time, particularly when kept at low temperatures. Lieske, however, reported that anaerobic forms are highly sensitive to drying, being unable to survive even for one day.

Influence of Temperature

The range of temperature within which many microbes are able to develop is comparatively wide. Most microbes begin to

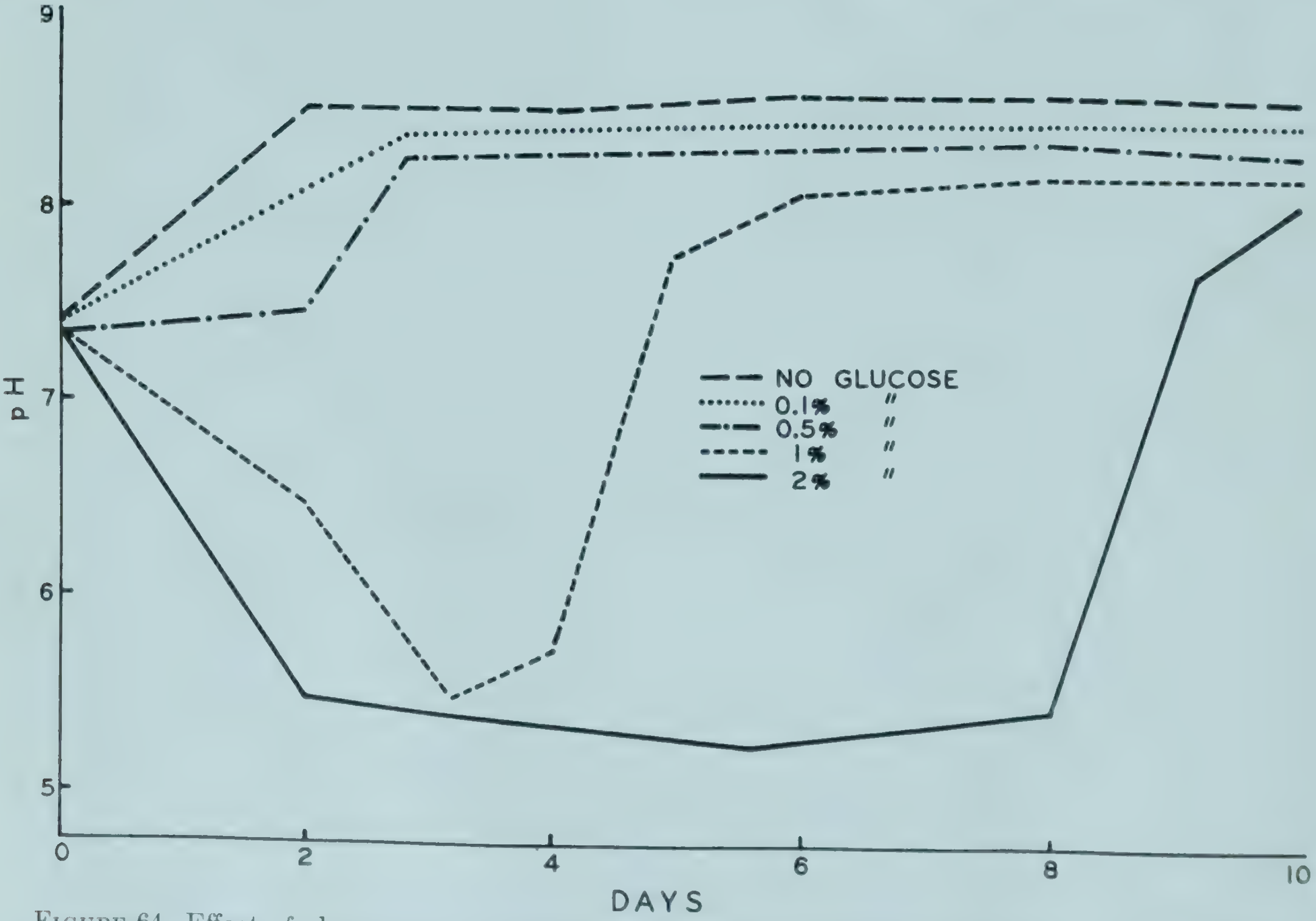


FIGURE 64. Effect of glucose concentration on pH (Reproduced from: Krassilnikov, N. A. 1950, p 206).

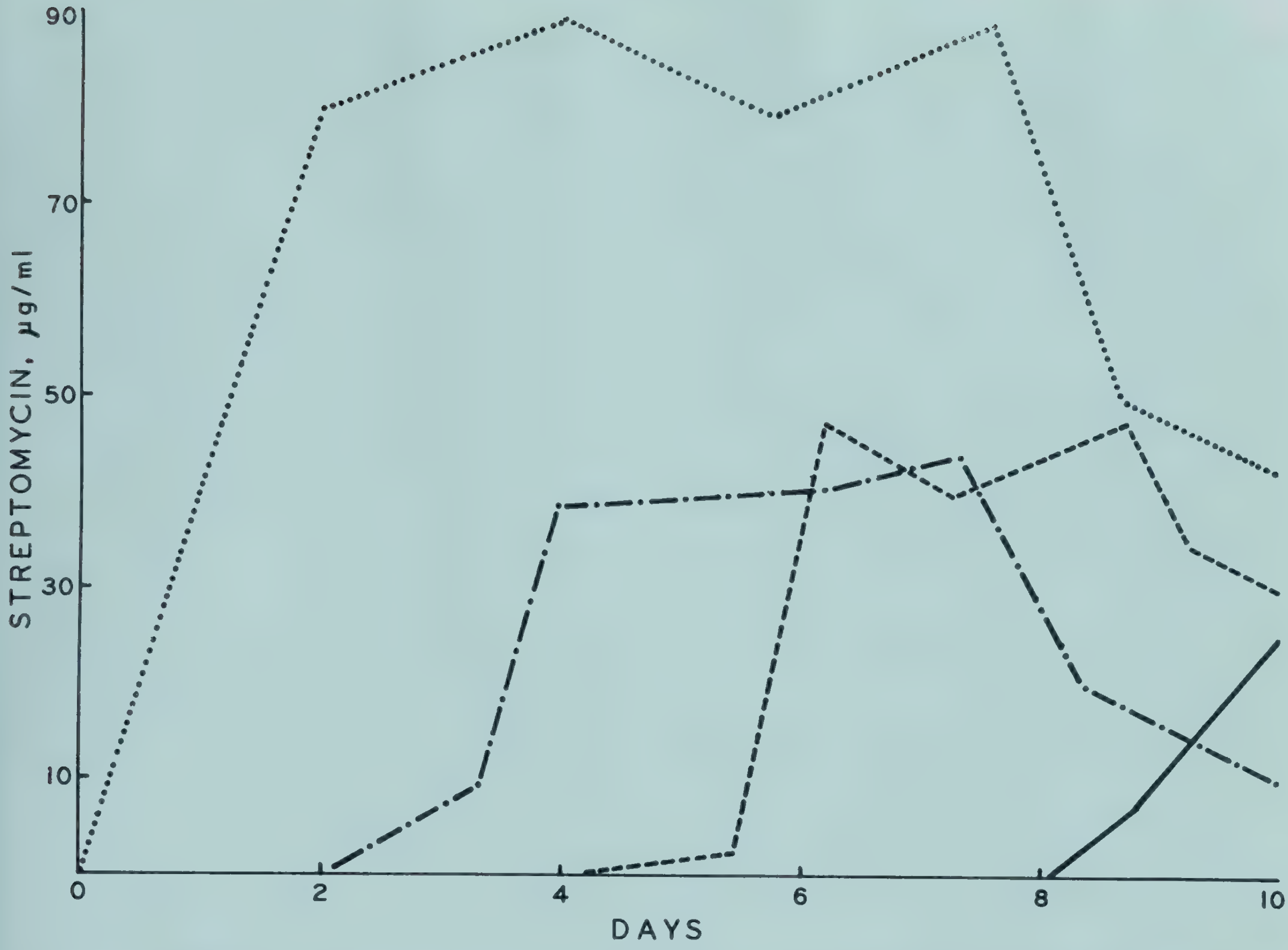


FIGURE 65. Effect of glucose on streptomycin production; key same as in Fig. 64. (Reproduced from: N. A. Krassilnikov, 1950, p. 206).

grow only above a certain temperature, although they may remain alive, without multiplication, at much lower temperatures. When the temperature reaches a point which is specific for each organism, growth begins. The rates of reproduction and of the metabolic reactions increase rapidly with a further rise in temperature up to a certain point, which is again specific for each organism. A still further rise in temperature leads to a drop in the rate of growth, until finally a point is reached at which growth stops.

According to Haines (1932), the ordinary saprophytic actinomycetes found in cold stores and in soil fall into two groups: 1. Those organisms that have their optimum temperature for growth at 37° C, their range of growth extending from 40 to 5° C, with a lower limit at just about 0° C. 2. Those that have a less sharply defined optimum temperature, growth being rapid at 20 to 30° C; corresponding to a lower optimum temperature is also a lower minimum temperature, growth being slow but good at 0° C, with a minimum between 0 and −5° C. The conclusion was reached that actinomycetes are probably of greater practical significance in modern trade practice in chilled meat, eggs, and possibly fruit than in well-frozen meat. The presence of active cultures is sufficient, without actual growth, to cause a "musty" taste in the stored product.

Three points are thus established in the temperature range for every organism: (a) a minimum or lower limit of growth; (b) a maximum or upper limit; and (c) an optimum at which growth is at its best. The optimum temperature may not be a sharp point, but may cover a comparatively wide range. Bacteria pathogenic to man and to warm-blooded animals develop within a much narrower range of temperature than do saprophytic bacteria. *M. tuberculosis*, for example, has its minimum at about 30° C, its maximum at 42° C, thus giving 12° C as a range of growth. On the other hand, many saprophytes have a range of growth of almost 40° C.

According to their temperature relations, microbes are usually divided into three groups:

1. Psychrophilic forms, with a minimum at 0°, an optimum at 15 to 20°, and a maximum at 30° C.
2. Mesophilic types, with a minimum at 9 to 30° C, an optimum at 28 to 38°, and a maximum at 43 to 50° C.
3. Thermophilic organisms, with a minimum at 40 to 49°, an optimum at 50 to 65°, and a maximum at 60 to 75° C.

The water-inhabiting organisms, particularly the marine types, and most luminescent bacteria comprise the first group. The pathogenic forms and most of the saprophytes belong to the second group. The third group includes certain bacteria which develop in hot springs and in soils of warm climates; many bacteria and actinomycetes are found in high-temperature composts. This classification is purely arbitrary, since there are no sharp lines of demarcation between these groups. The above limits of temperature are not constant, and depend on the composition of the medium, concentration of nutrients, and other environmental conditions.

When the temperature is reduced below the minimum or raised above the maximum, growth of the organism stops. An injurious effect follows only a considerable change in temperature. This effect is more marked on the upward scale than on the downward scale. Microbes are much more resistant to temperatures below the minimum than to those above the maximum.

Lowest possible temperatures are usually not sufficient to kill microbes. Even temperatures of liquid air were not sufficient to kill staphylococci and *M. tuberculosis*. However, some bacteria are rapidly killed at 0°. Repeated freezing and thawing have a more deleterious effect than freezing alone; different organisms vary in this respect. Bacteria and spores in a dry state are preserved by liquid air. The mycelium and spores of certain fungi were not killed even by −110°, whereas the mycelium of other fungi was destroyed readily at low temperatures, the spores being more resistant.

Most bacterial cells, except the thermophilic forms, are killed at a temperature of 56° C for 1 hour; at 60° C, 10 minutes is sufficient, whereas at 80° C only 1 minute will kill these bacteria. The temperature and time necessary to kill bacterial cells vary with the kind of bacteria and with the composition of the medium; a longer period is required for bacteria in organic media than for suspensions of bacteria in water or in salt solution.

The optimum temperature for growth of most of the actinomycetes usually falls between 23 and 37° C. Certain actinomycetes are able to grow at temperatures lower than 20° C, whereas some prefer temperatures of 20° to 23° C. The more common forms are readily destroyed at the higher temperatures, the resistance of the spores being only slightly greater than that of the mycelium. When a culture is kept for 10 minutes at 70° C, not only the mycelium but also the spores lose their viability.

The actinomycetes are able to withstand much higher degrees of dry heat than of moist heat. Knorr (1933) found that exposure of soil to dry heat at 180 to 200° C may not be sufficient to destroy all actinomycetes. Some actinomycetes can be adapted to grow at higher temperatures. Lieske, for example, was able, after a few transfers, to grow his culture *Nocardia* 74 at 48° C.

whereas the original upper temperature limit for this culture was 42° C.

Further studies on the effect of temperature and humidity upon the growth and death-rate of spores and mycelium of various streptomyces have been made by Jagnow (1957).

Among the actinomycetes, a special group stands out in relation to temperature conditions. These are the thermophilic actinomycetes that are capable of growing at temperatures of 50 to 65° C. These organisms occur abundantly in manure composts, in heaps of hay, and in pasteurized cheese.

Gilbert isolated several thermophilic forms from various soils and included them in one species, *A. thermophilus*. The optimum temperature for growth was 55°, with a maximum at 60° C. Most strains ceased to grow at 45°, although some could be adapted to grow on agar media at 37° and even lower temperatures. Gelatin was slowly liquefied. Miehe looked upon the thermophilic actinomycetes as the characteristic organisms inhabiting the decomposing masses of plant material under high-temperature conditions. The actinomycete spores lost their vitality rapidly, especially on agar media, but survived on hay particles. One organism, designated as *A. thermophilus* Berestnew, grew well at 40 to 50° C, more slowly at 30°, and not at all at 25 and 60° C. The manner of spore formation of this organism suggests that it was a member of the *Micromonospora* group. Schütze reported the presence in decomposing clover hay of representatives of two types of thermophilic actinomycetes, one of which was designated as *A. thermophilus* Berestnew and the other as *A. monosporus* Lehmann and Schütze. The latter may also be considered a member of the *Micromonospora* type.

Further studies on the germination of heat-resistant spores of *M. vulgaris* led Erikson (1955a) to conclude that heat activation at 100°C for 1 minute enhanced the initial germination rate of the spores within the first 3-hour period. When the spores have been subjected to temperatures of 100°C for more than 1 hour, sectored colonies showing loss of aerial mycelium and impaired viability frequently developed. Erikson (1955b) further reported that a pathogenic partially acid-fast culture of *N. sebivorans* and a closely allied form were capable of withstanding exposure to 90°C for 10 minutes when dispersed in phosphate buffer suspension. Subjection to heat treatment (90°C for 1 minute) of a blood culture belonging to the *S. albus* group increased the autolytic tendency of the culture and affected the type of sporophore produced during the first generation.

Waksman, Gordon, and Hulpoi made a study of the occurrence of actinomycetes in high-temperature composts, as will be shown in Chapter 16. Waksman and Corke examined the classification of the thermophilic actinomycetes and came to the conclusion that these microbes represent two distinct groups of the *Streptomyces* and *Thermoactinomyces* types. Temperature-growth relationships of *Micromonospora* have been studied in detail by Erikson and Webley.

Effect of Drying

Microbes vary greatly in their sensitivity to drying. Some are highly sensitive and cannot resist drying more than a few minutes or a few hours; others remain alive for many years. The composition of the medium is of great importance in this connection; the presence of protein in the medium greatly increases the period of resistance to drying. Bacteria remain alive in normal air for a much shorter time than in dry air, because of the moisture content of the former. In dry air, many bacteria remain alive for years. Bacterial spores resist drying much more readily and for a longer period than do vegetative cells. The mycelium of most fungi is readily destroyed on drying, while the spores are killed only after prolonged drying, the period varying with the species. The greater resistance of the spores is due to their lower water content; the mycelium of a *Penicillium* was found to contain 87.6 per cent of water, and that of the spores 38.9 per cent.

Actinomycetes are very abundant in dry soils and are, in general, markedly resistant to drying. Acosta kept a culture of an actinomycete (*A. invulnerabilis*) alive in a fully dry state for 9 years. Berestneff inoculated a culture of *S. violaceus* on sterile rye straw and allowed it to grow until sporulated. After being kept in a dry state for 10 years in the laboratory, it was still alive.

Lieske prepared dry cultures (on sterile filter paper) of *Streptomyces*, *Nocardia*, and *Actinomyces* in a desiccator, over dry $CaCl_2$ and sulfuric acid. The cultures were alive after 18 months. Different organisms differ greatly, however, in their ability to survive drying under ordinary atmospheric conditions.

Krassilnikov (1938) emphasized the remarkable ability of actinomycetes to survive, under most unfavorable conditions, for very long periods.

Influence of Light

Actinomycetes do not need light for their activities. In fact, strong light, especially when prolonged, has an injurious effect upon their development. The effect of light depends to a great degree upon the medium in which the organisms are grown; the cells are usually more resistant in milk than are those in bouillon. The resistance of the organisms to light is greater in a dried than in a moist condition. The injurious action of light increases with the intensity of the source of light. Sunlight acts only on the surface of solid media or in the air to which the organisms are exposed. In liquid media, those organisms which are subjected to the greatest intensity and are only slightly protected, are destroyed.

The red and orange rays of the light spectrum, as well as the infrared, or heat rays, have no effect upon the growth and activities of microbes. Blue and violet, and especially the ultraviolet, are the most injurious rays of the spectrum. Various explanations of the mechanism of the microbicidal action of ultraviolet radiations have been offered. It is known that microbes absorb the lethal rays and that the proteins have absorption bands between 2480 Å and 2710 Å. The effective radiation for sterilization is in the region of wave lengths of 2800 Å to 2500 Å. The capsulated organisms are most susceptible; the sporulating organisms are most resistant.

When a microbial culture is suddenly brought to light, the protoplasm contracts and a partial dehydration of the cell contents takes place under the influence of the rise in temperature. When the culture is returned to darkness, the reverse takes place. Ultraviolet radiation produces an enormous contraction; the vacuoles are reduced and may even disappear, followed by plasmolysis.

The antibacterial action of radiations, comprising both the long ultraviolet (>3500 Å) and short visible (<4900 Å) rays, has recently attracted considerable attention from the point of view of destruction of undesirable organisms and development of mutants from desirable ones. The region of 3500 to 4900 Å is particularly effective. It was concluded that the extended nature of the killing curve suggests the production of some toxic substance, or the destruction of some essential compound in the cell, the effect of which, up to a certain limit, does not permanently destroy the ability of the cell to divide and develop further. Wave lengths shorter than 3000 Å are most efficient at 2650 Å, close to the wave lengths at which nucleic acids act as most active absorbents. The phenomenon of photoreactivation greatly influences the killing effect of ultraviolet light (Kelner, Pittenger and McCoy).

Action of Stimulants and Poisons

Poisonous substances usually affect microorganisms in one of the following ways: 1. At certain low concentrations, which are definite for each substance, there is no appreciable action. 2. At somewhat higher concentrations, these substances may exert a stimulating effect upon growth. 3. A further increase in concentration of the substance produces a bacteriostatic effect upon growth. 4. Still further increases have a microbicidal effect. There is a sharp line of demarcation between stages 2 and 3, but hardly any between 3 and 4, because a concentration which has a bacteriostatic effect may become bactericidal on prolonged action.

With regard to their antimicrobial effects, chemical substances are looked upon as indifferent agents, as stimulants, or as poisons, depending entirely upon the activity of the particular substance.

Microbial stimulants have been classified into three groups: (a) chemical stimulants, (b) effective nutrients, including oxygen, and (c) effective metabolic products. When present in very small doses, toxic substances may act as stimulants; fluoride, for example, increases the production of zymase by yeasts. The chemical stimuli are often differentiated between those that affect the germination of spores and those that favor growth and reproduction.

Kruse proposed the following rules for the toxic action of poisons upon microbes: 1. The bacteriostatic or bactericidal action of a poison increases with the concentration. 2. With an increase in the amount of inoculum, the toxic effect of the medium decreases or disappears. 3. The weaker the nutritive power of the medium, the greater is the toxicity; it is strongest in water and weakest in protein solutions. 4. The poison has a greater bactericidal action at higher temperatures.

The action of a poison and the resistance of the cell depend upon the rapidity with which the substance passes through the plasma membrane and penetrates into the cell. The fat-soluble substances are, therefore, more poisonous. The strongest poisons are the metallic salts. $AgNO_3$ in a concentration of 1:800,000 will kill most bacteria, and $HgCl_2$ is still more toxic; they are most soluble in ether, alcohol, and fats. Iodine and other substances, such as alcohols, chloroform, ether, and CS_2 behave in a similar manner. Copper salts are not soluble in lipoids, but tend to form complex organic compounds with the cells.

Of the various microbial poisons, the following groups are found to be particularly effective: salts of certain heavy metals, including silver, gold, mercury, and copper. The last two have found an extensive application as fungistatic and fungicidal agents. Iron has little antimicrobial activity, and lead, nickel, and zinc are not active as poisons. Mineral acids, particularly those of the halogen group, have a powerful action. Of the soaps, only the salts of saturated fatty acids are poisonous. Potassium permanganate and peroxides have a strong effect. Formaldehyde is effective. Ethyl alcohol is active only in certain dilutions with water. Of the aromatic compounds, phenol and its derivatives, especially the chlorinated and brominated compounds, are most important.

A number of other organic compounds, as substituted ammonium salts, salicylanilide, dichlorodihydroxybenzomethane, and many others, have found extensive use as fungicides. The dyes may be added to the list of bactericidal agents.

In recent years, a new type of antimicrobial agent has gained universal recognition. It comprises the antibiotics, or compounds of microbial origin. Their effect upon actinomycetes are discussed in detail in Chapters 14 and 15.

CHAPTER 8

Mineral Metabolism and Effect of Salts on Growth

Mineral elements play a highly important role in the growth of microorganisms. They function both as essential nutrients for cell synthesis and as regulatory mechanisms for various transformations that take place in the living systems. The composition of most of the synthetic media used for the growth of actinomycetes bears out this fact. Aside from the required sources of carbon and nitrogen, actinomycetes require phosphorus, sulfur, iron, potassium, magnesium, and certain other inorganic elements. In some cases, as in the production of certain antibiotics, pigments, and vitamins, such elements as potassium, calcium, chlorine, manganese, cobalt, and zinc play most interesting parts in the biochemical reactions involved.

In most of the earlier studies on the effect of inorganic salts on the growth of actinomycetes, complex organic media were used. Münter (1916), for example, used a medium containing blood protein, gelatin, and agar. He still observed that potassium and sodium salts, when used in 5 per cent concentrations, are favorable for growth but not for sporulation. He further noted that the addition of small amounts of Ca, Ba, and Sr were favorable for growth and sporulation; higher concentrations were injurious.

With the recognition of the important role of actinomycetes as producers of antibiotics and vitamins, there has been an ever growing interest in the role of mineral elements in their nutrition. It was soon established that the great majority of actinomycetes, like other microorganisms, grow at rather low concentrations of salts. Some, however, are able to tolerate very high concentrations.

Essential Nutrients

Among the organisms studied most extensively from the point of view of mineral requirements, *S. griseus* occupies a leading place. A chemically defined medium is more desirable than a complex organic medium for investigating metal requirements for nutrition and for antibiotic production.

In a study of the mineral requirements of *S. griseus* by Chesters and Rolinson, a chemically defined medium was used. It was made metal-deficient by treatment with chloroform solutions of diphenylthiocarbazone at pH 7.3 to remove zinc and copper and with 8-hydroxyquinoline at pH 5.2 to remove iron and at 7.3 to remove manganese. Media from which the metals were omitted singly were compared to media in which they were included. When zinc was omitted, the medium supported the synthesis of only 75 mg of cell material per 100 ml of medium. Addition of zinc equivalent to 1 part per million of medium permitted maximum growth (550 mg/100 ml) and maximum streptomycin production. Further increases in the concentrations of zinc resulted in decreased antibiotic production, so that only 50 per cent of the maximum antibiotic production took place with 50 parts per million of zinc. The addition of 0.05 part per million of copper resulted in optimum growth and antibiotic production. Iron affected growth and formation of the antibiotic at different levels: optimum concentration for growth was 0.3 part per million, whereas optimum antibiotic production required 1.0 to 2.0 parts of copper. No effect of manganese could be observed over a range of 0.005 to 50 parts per million.

Thornberry and Anderson developed a synthetic medium for streptomycin production which contained, in addition to carbon and nitrogen sources, potassium, magnesium, zinc, iron, copper, and manganese. The effect of a number of metals was studied by adding the metals to, or omitting them from, this medium. Growth was estimated by comparison with that obtained in a complex medium. The conclusion was reached that potassium, magnesium, zinc, and iron were needed for good streptomycin production and supported excellent growth. Manganese stimulated antibiotic production but had no effect on growth. Calcium had no effect on either antibiotic production or growth.

Temple found that magnesium and potassium exerted the most noticeable effect on both growth and antibiotic production. Iron had a lesser effect on growth but was required for high streptomycin yields. A modification of Thornberry's medium to contain ammonium citrate and inositol required additional calcium. If tap water were used in place of distilled water, no additional calcium was needed. Saunders and Sylvester reported that traces of zinc, copper, iron, magnesium, and manganese were necessary for optimum streptomycin production. Principe and Thornberry found that the addition of cobalt in a concentration of 0.003 M increased streptomycin production by 83 per

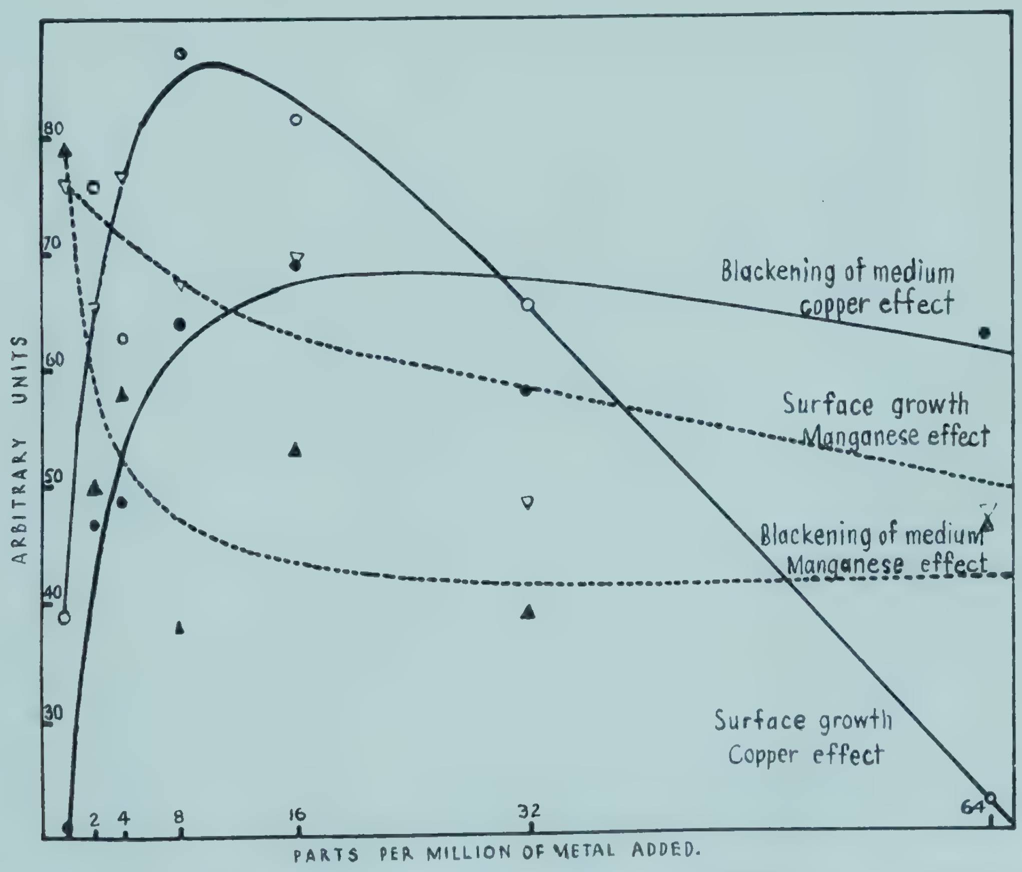


FIGURE 66. Effect of copper and manganese on surface growth and pigment production by *S. griseus* (Reproduced from: Spilsbury, J. F. Brit. Mycol. Soc. Trans. 31: 215, 1948).

cent. Growth was not affected at concentrations lower than 0.009 *M* but was inhibited at that and higher concentrations.

Chalupka (1957) found that of the various ions tested (K, Na, Mg, Ca, Fe, and Zn), only K (0.05 *M*) caused a significant increase in the mycelial mass of *S. griseus* and in protease formation. According to Saunders and Sylvester, Zn, Cu, Fe, Mg, and Mn were necessary for optimum streptomycin production.

Metals had a beneficial effect on growth and streptomycin production also in complex media. Spilsbury used a peptone-meat extract medium. The ions of lead, tin, uranium, vanadium, cerium, strontium, chlorine, iodine, and fluorine had no appreciable effect at any concentration employed. Slight stimulation of growth appeared to result from ions of bromine at 2 parts per million, zirconium at 10 parts per million, and all concentrations of molybdenum employed. Bismuth and lithium were slightly toxic at 2 parts per million; aluminium, cobalt, and nickel were toxic at 10 parts per million; cadmium proved to be extremely toxic at all concentrations employed. The metals most likely to prove worthy of investigation were considered to be copper, iron, manganese, zinc, and molybdenum, together with the major salt constituents, notably sodium nitrate, potassium phosphate, and magnesium sulfate. Tryptophan was shown to be essential in the early stages of development. It is of further interest to note that whereas copper and iron caused an increase in growth,

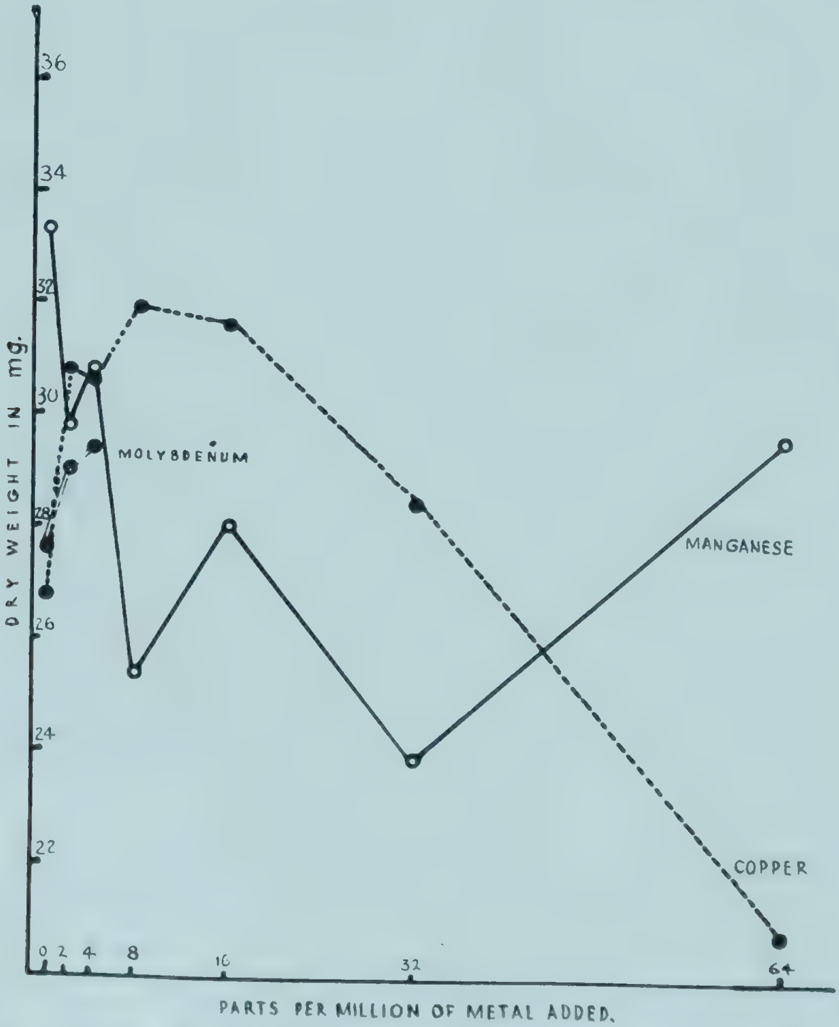


FIGURE 67. Effect of copper, manganese and molybdenum on dry weight of *S. griseus* (Reproduced from: Spilsbury, J. F. Brit. Mycol. Soc. Trans. 31: 217, 1948).

only copper yielded an increase in streptomycin production. Manganese and zinc, however, caused a decrease in antibiotic formation (Table 30).

TABLE 30

Effect of metals on the growth, streptomycin production, and pH of S. griseus (Spilsbury)

Treatment	7 days			12 days	
	Dry wt, gm	Streptomycin, μg/ml	pH	Streptomycin, μg/ml	pH
Control	2.51	125	7.16	100	7.70
Copper, 50 ppm	4.64	80	7.41	130	8.00
Copper, 10 ppm	4.32	150	7.59	100	8.10
Zinc, 50 ppm	0.94	—	7.24	20	7.50
Zinc, 10 ppm	1.41	65	7.27	15	7.70
Manganese, 50 ppm	0.98	115	7.20	—	7.00
Manganese, 10 ppm	2.77	120	7.06	—	7.60
Iron, 50 ppm	1.58	115	6.88	—	7.05
Iron, 10 ppm	2.29	110	7.02	70	7.70
Cu × Zn	2.84	145	7.06	150	8.10
Cu × Mn	3.71	170	7.35	130	8.00
Cu × Fe	4.10	130	7.25	200	8.20
Mn × Zn	1.72	80	7.24	—	7.00
Zn × Fe	1.32	40	7.10	—	—
Mn × Fe	2.25	55	7.20	—	7.30
Cu × Zn × Fe	4.26	90	7.19	—	8.15
Cu × Fe × Mn	4.06	125	7.46	200	8.25
Fe × Mn × Zn	1.90	45	7.16	50	7.70
Cu × Mn × Zn	3.59	110	7.25	110	8.10

Using a basal medium containing glucose in nutrient broth, Woodruff found increased streptomycin production in surface cultures of *S. griseus* to which sodium chloride, ferrous sulfate, and zinc sulfate were added. Maximum production was affected by the ratio of the supplements and the source of the water. Zinc alone resulted in rapid pellicle formation but decreased streptomycin production, whereas the addition of iron caused a decrease in growth but an increase in antibiotic production.

Streptomycin production by *S. bikiniensis* was affected differently, as shown by Johnstone and Waksman. The addition of ferrous sulfate had no effect, but the yield of the antibiotic was increased by the addition of zinc sulfate to a medium containing meat extract, peptone, glucose, sodium chloride, and tap water. When both zinc and iron were added at the same time, no noticeable effect was obtained. Changes in sodium chloride concentration also influenced antibiotic production, the most effective concentration being 1 per cent. By adding sodium chloride to a complex medium containing soybean meal, Rake and Donovick obtained increased streptomycin production by *S. griseus*. Eiser and McFarlane found that complex media with and without sodium chloride supported the same amount of growth on a dry weight basis, with no difference in the residual glucose, ammonia, or amino nitrogen. In the absence of sodium chloride, streptomycin accumulated in the mycelium, whereas in its presence the streptomycin diffused more rapidly into the culture medium. Since the cation and anion could be replaced by other closely related ions in the Hofmeister lyotropic series, still yielding similar results, the authors ascribed the results to the effects on membrane permeability rather than to osmosis.

The concentration of iron is an important factor. Rao reported a 3.6-fold increase in streptomycin production as a result of the addition of iron to the medium. Asai *et al.* studied growth and streptomycin production in a complex, iron-rich medium, and found that both were inhibited at iron concentrations greater than 0.015 per cent w/v. They attributed the inhibition to a colloid-chemical phenomenon in which an iron gel covered the mycelium, with the result that the organism was unable to take up nutrients or oxygen from the medium.

The production of other antibiotics is also affected by the presence of metal ions. Lechevalier found that no neomycin was produced by *S. fradiae* in a medium containing peptone, beef extract, glucose, sodium chloride, and distilled water. When tap water was substituted neomycin was formed. The addition of zinc, even as low as 1 part per million to media, resulted in antibiotic production even in distilled water media. Iron, manganese, copper, aluminum, calcium, and magnesium, had no such effect. Lechevalier was able to show a requirement for traces of potassium, magnesium, iron, and calcium for optimum neomycin production in media containing glutamic acid and glucose.

Dulmage also reported that in a synthetic medium containing glutamic acid and glucose to which metal salts were added, it was necessary to have potassium, magnesium, iron, zinc, and calcium for both growth and neomycin production. When glutamic acid was used as the only source of carbon and nitrogen and the medium treated with 8-hydroxyquinoline or ethylenediaminetetraacetic acid to render it metal-deficient, a requirement for iron, calcium, magnesium, and zinc for neomycin production by *S. fradiae* was reported by Mohan and Nickerson. These investigators were able to show a requirement for calcium and magnesium for growth, about a 50 per cent decrease in growth when iron was omitted, and no effect on growth when zinc was omitted from the medium.

Acker and Lechevalier made a study of some nutritional requirements of *S. griseus* for growth and candicidin production. A synthetic medium was used; it was made metal-deficient by calcium carbonate coprecipitation. Essential requirements for potassium, magnesium, iron, and zinc were demonstrated. No effect of manganese on either growth or antibiotic production was obtained.

In a study of the role of iron for the production of grisein by *S. griseus*, Reynolds and Waksman demonstrated that as the concentrations of iron were increased, grisein activity increased logarithmically. This was due to the fact that iron is a part of the grisein molecule. When iron was added to a solution of grisein in concentrations of 4 gm/l as ferrous sulfate, partial inactivation of the antibiotic resulted. Complete inactivation of grisein occurred when 25 gm of ferrous sulfate were added per liter of grisein solution. Iron appeared thus to play a quantitative role. Zinc had no effect on grisein production.

Kelner and Morton reported that iron has a similar effect on the production of actinorubin. In a tryptone-glucose medium supplemented with mineral salts, ferrous sulfate added in concentrations of 5 mg/l resulted in increased antibiotic production, whereas concentrations of 20 mg or more caused a decrease in yield.

In various other studies the production of antimicrobial activity by three strains of streptomycetes was found to be influenced by manganese, zinc, iron, and copper. Concentrations lower than 2 mg/l were ineffective. Copper was inhibitory at a concentration of 10 mg/l. A complex medium was used in these investigations.

The addition of different concentrations of sea water to a glucose-peptone-yeast-extract medium resulted in an increase in soil isolates (Jann *et al.*). Two streptomyces cultures gave increased antibiotic production on addition of sodium, potassium, calcium, and magnesium as chlorides, sulfates, or nitrates. With a third culture all of the above metals, especially calcium caused an increase in production of antibiotic. In the case of a fourth culture, only calcium and sea salts gave an increase in antibiotic yield.

The importance of cobalt in the production of vitamin B_{12} is due to the presence of this metal in the vitamin molecule. The need for cobalt was demonstrated before the structure of this vitamin was known. Hendlin and Ruger found that cobalt became a limiting factor for vitamin production by *S. griseus*, even in a complex medium. Cobalt, added as $Co(NO_3)_2 \cdot 6H_2O$, caused a 3-fold increase in vitamin B_{12} production, compared

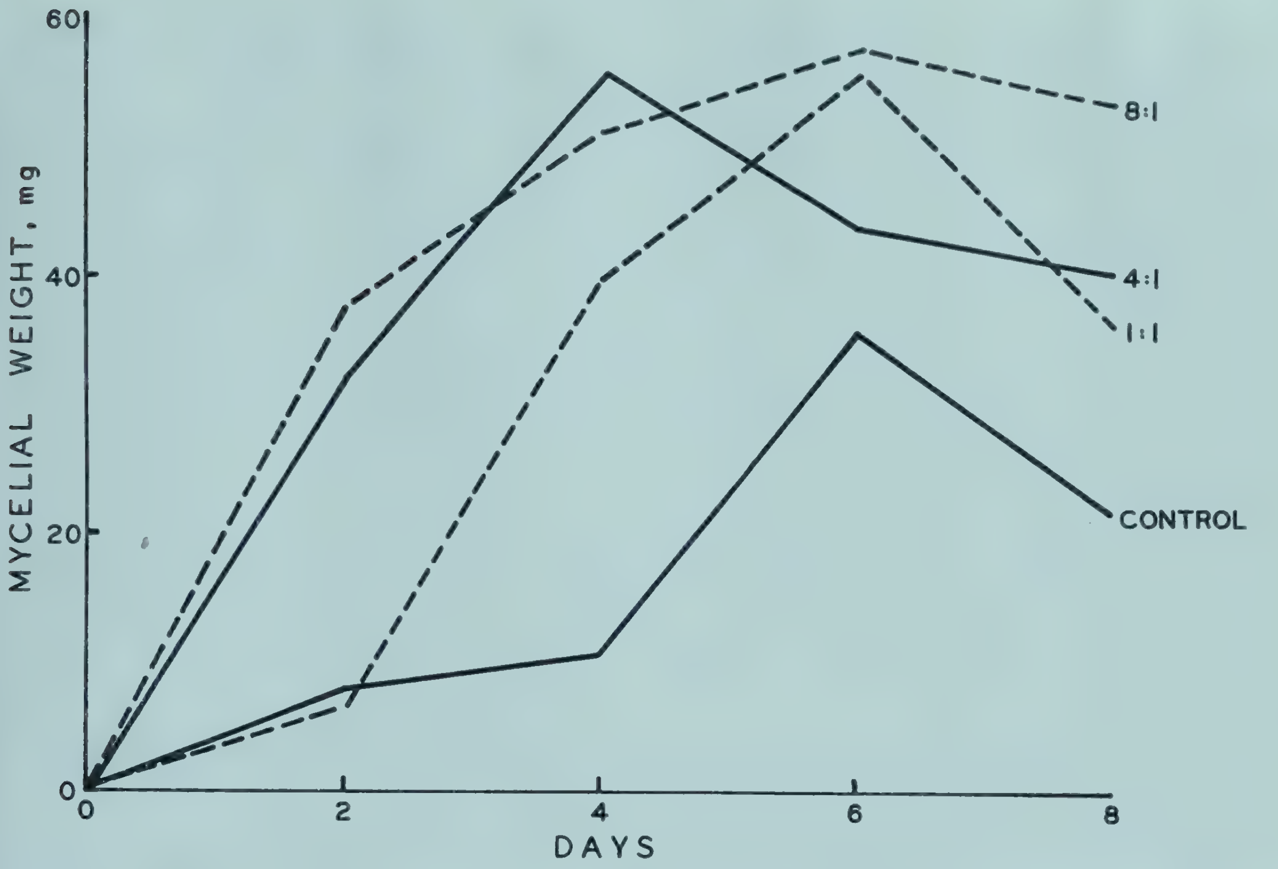


Figure 68. Effect on streptomyces mycelial growth produced by various concentrations of compost soil extract (Reproduced from: Spicher, G. Zentr. Bakteriol., Abt. 2, **108**: 579, 1955).

to media from which cobalt was omitted. Levels of 1 to 2 parts per million cobalt yielded maximum results; levels of 20 to 50 parts per million were toxic. Cobalt has since been used routinely in culture media for screening and for production of vitamin B_{12}. The form of cobalt and the concentration varied according to the individual investigator and the media used. A cobalt-tolerant culture of *Saccharomyces cerevisiae*, developed by Perlman and O'Brien, added to a soybean meal-glucose medium inoculated with *S. griseus* could be utilized as a source of cobalt for vitamin B_{12} production more efficiently than inorganic cobalt added as the nitrate. The increased efficiency was noted over a concentration range of 0.01 to 0.1 μg/ml, but not at higher levels. (See also Burton and Lochhead, Charney, Dulaney and Williams, Kojima and Matsuki, Principe and Thornberry, Smith *et al.*). Radioactive vitamin B_{12} was produced by *S. griseus* from cobalt^{60} (Chariet *et al.*).

The necessity for cobalt to give increased yields of vitamin B_{12} by *S. olivaceus* and other organisms is now well established (Hall *et al.*). Copper, at concentrations of 82 parts per million, almost completely inhibits vitamin B_{12} synthesis; at concentrations of 133 parts per million, it permits only slight growth of the organism. No effect on growth or vitamin production was observed at concentrations of 56 parts per million. Strips of metallic copper in the medium inhibited vitamin B_{12} synthesis. Strips of stainless steel, tin, aluminum, iron, or lead placed in the medium showed no effect.

In addition to its effect upon the production of vitamin B_{12}, cobalt exerts other effects upon the growth of actinomycetes. Hickey and Tresner reported that the addition of 2 mg of $CoCl_2 \cdot 6H_2O$ to a modified Bennett's medium greatly favored the rate and extent of sporulation of *S. fradiae* and of a number of other streptomycetes. Sporulation was suppressed when the cobalt was replaced by zinc or iron. Growth of *S. scabies* was inhibited by aluminum ions in concentrations of 20 or more parts per million (Gries). This inhibition could not be reversed by the addition of magnesium.

The addition of metals to the medium also influences greatly pigment production. This is true of ferroverdin, a green iron-containing pigment produced by a streptomyces. Zinc sulfate concentrations of 0.001 per cent increase the rate of pigment formation in strains of red-yellow-pigment-forming streptomycetes (Chain *et al.*). Omission of magnesium or calcium from the medium reduced the amount of diffusible pigment but omission of zinc or iron did not have any effect on pigment production by *S. fradiae* (Mohan).

Stapp and Spicher and Spicher observed that soil extract, or ash of soil extract, added to synthetic nutrient solutions, gave increased growth of a group of streptomycetes (Fig. 68). The increase in growth was nearly proportional to the amount of ash added. The media were purified by various procedures, including sulfide precipitation and use of calcium carbonate and 8-hydroxyquinoline. When added to the medium in certain concentrations and combinations, various metals also yielded increased growth over metal-deficient controls. The most effective metals and concentrations were: iron at 0.05 mg per 100, manganese at 0.0005 mg per 100, and zinc, copper, and molybdenum at 0.005 mg per 100. Combinations were more effective than single additions, the most effective being iron-zinc, iron-manganese-zinc, and iron-manganese-zinc-molybdenum (Fig. 69).

Heim and Lechevalier used a chemically defined medium made metal-deficient by treatment with activated chromatographic alumina. The effect of iron, zinc, manganese and calcium on the growth of eight strepto

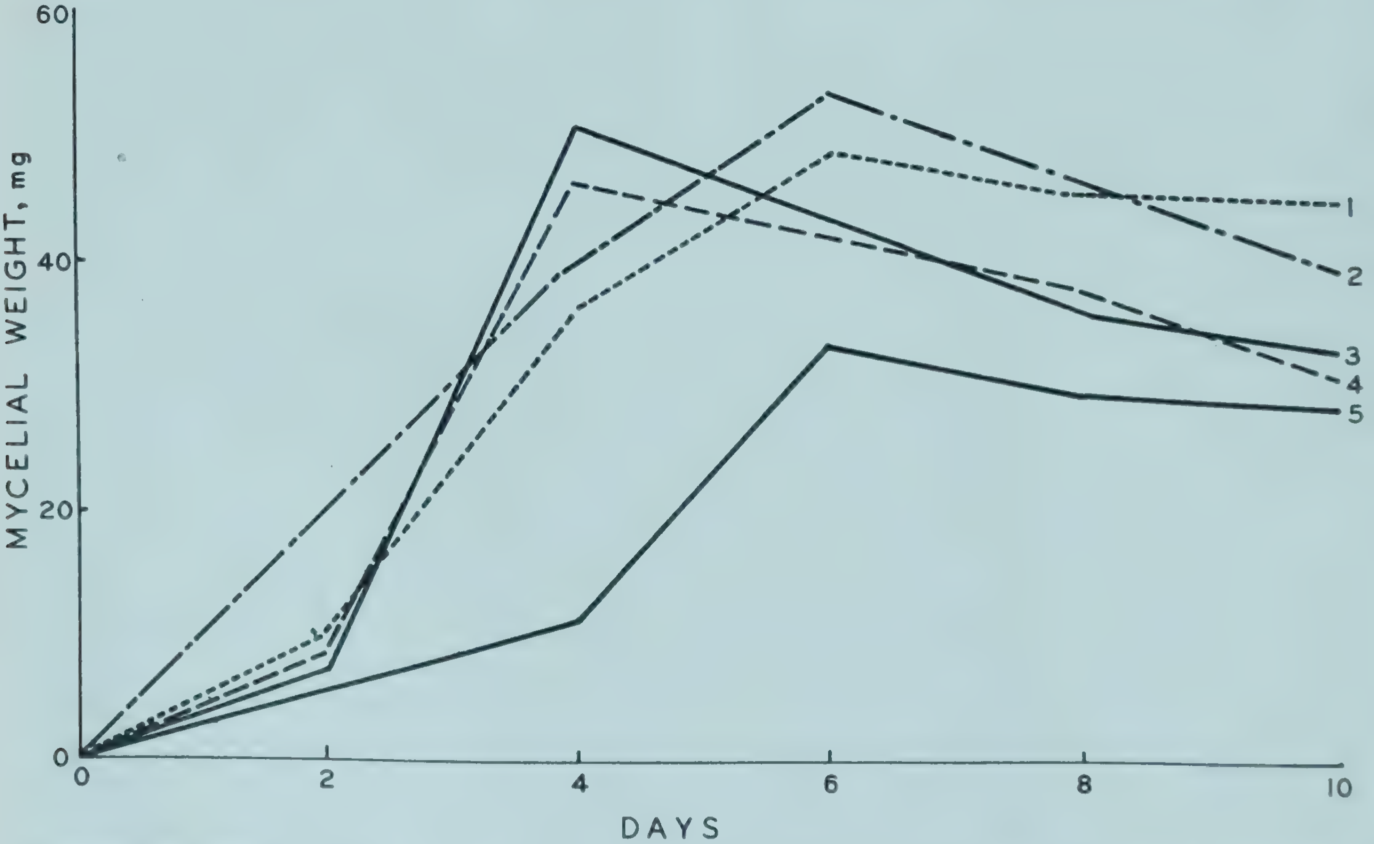


FIGURE 69. Effect on streptomyces mycelial growth produced by various soil extracts: 1. field soil 2. compost soil, 3. field soil, ash of extract, 4. compost soil, ash of extract, 5. control (Reproduced from: Spicher, G. Zentr. Bakteriol., Abt. 2, 108: 580, 1955).

myces cultures was studied. The metals were then added singly and in all possible combinations at concentrations of 3 parts per million. Growth was used as the criterion of effect, and the results were analyzed statistically. Iron and zinc were beneficial for the growth of all eight cultures investigated. Calcium was beneficial for six of the eight; *S. lavendulae* and *S. aureofaciens* were unaffected. Manganese was effective for only *S. coelicolor*. An iron-zinc interaction was beneficial in all cases.

Calcium was found to retard the onset and rate of lysis of *S. fradiae* in concentrations as low as 1.5 parts per million. It also enhanced the utilization of glycine and glutamic acid in the medium. Mohan observed that increasing concentrations of calcium chloride in the medium enhanced the utilization of glutamic acid. Magnesium could not be replaced by manganese in the medium and its omission resulted in almost no growth of *S. fradiae*. The effect of copper on four streptomyces cultures was investigated by Heim; growth of *S. fradiae* decreased 55 per cent when copper was omitted, but there was no effect on *S. griseus*, *S. rimosus*, or *S. lavendulae*.

Due to the specific effect of iron, Heim *et al.* analyzed for the presence of cytochromes. In a survey of 13 cultures five were found to contain only a *b* type cytochrome, and eight contained both *b* and *c* types. It was suggested that the importance of the cytochromes as respiratory pigments and the presence of iron in the molecule may explain, at least in part, the importance of iron in the metabolism of the streptomyces.

Potassium, magnesium, zinc, iron, copper, and calcium are thus shown to be required for most, if not all, of the phases of the metabolism of actinomycetes. Certain metals, such as cobalt for vitamin B_{12} production and iron for the grisein molecule, are required for incorporation into specific molecules. A study of sodium requirement indicated a special effect of this element on the diffusion of streptomycin into the culture medium of *S. griseus*. The presence of this element does not appear to be a general requirement for nutrition of actinomycetes. Manganese is not required generally for growth or antibiotic production; in some cases it was even slightly inhibitory. The inhibiting effect of aluminum ions was also reported. This would appear to be not a qualitative effect but rather a quantitative one, since Heim could not observe any inhibition due to its presence in a medium treated with chromatographic alumina.

The effective concentrations of various metals in the nutrition of most actinomycetes vary according to conditions under which the organism is grown or according to the specific effect required, such as antibiotic or vitamin production. Metals which exert a beneficial effect at some concentrations may be inhibitory at higher concentrations. This is common with cobalt in vitamin B_{12} production and iron in the formation of some of the antibiotics.

In a study of the formation of chlortetracycline and bromtetracycline by different mutants of *S. aureofaciens*, some were found to be independent of the Cl concentration over a range of 0.02 to 10.0 stoichiometric equivalents, whereas the formation of chlortetracycline by other mutants depended on the Cl concentration over the above range.

Gallicchio and Gottlieb (1958) studied the effects of the microelements Zn, Fe, Mn, Mo, Co, Cu, B, and Ga and the macroelement, Mg, on growth and chloramphenicol production by *S. venezuelae*. Elimination of Zn or Fe from the mixture added to a $CaCO_3$-treated synthetic medium resulted in the suppression of chloramphenicol production. No one mineral in the synthetic medium supported production of the antibiotic, but the addition of Zn with Fe had a favorable effect. A larger concentration of Mg was required for growth than for chloramphenicol production. Mn could replace Mg in the growth requirement, but only a Mn concentration of 10^{-2} M allowed any chloramphenicol production. In a medium containing optimum concentrations of Zn, Fe, and Mg, the presence of the other microelements studied, singly or in groups, had little effect on growth or the production of the antibiotic.

Metals are common contaminants of most of the media used for the growth of actinomycetes. Complete removal of some metals, such as calcium, copper, and iron, is virtually impossible. A positive reaction in any medium indicates, however, a qualitative effect of the particular metal on the particular reaction. More refined experiments are required to explain the enzymatic basis for the reactions observed.

The effect of minor elements on the growth and nutrition of actinomycetes has been studied further by Perlman (1949), and by numerous other investigators. Their effect on antibiotic production was examined by Sanchez-Marroquin and Arcimega.

Effect of Salt Concentration

As pointed out elsewhere, actinomycetes have been reported to occur in sea water and in sea bottoms. According to Kober, *S. oligocarbophilus*, grown in a 0.5 to 0.6 M NaCl solution, produced fewer but larger colonies than in NaCl-free solutions. Although $MgSO_4$ is not essential for growth, it had a favorable effect even in fairly high concentrations. Calcium was not essential for growth, but its absence had an injurious effect unless magnesium was present. Potassium, in concentrations of 0.5 per cent KCl, was injurious; this effect was neutralized by high concentrations of $MgSO_4$. *S. oligocarbophilus* is noticeably hallophilic and could tolerate 25 per cent $MgSO_4 \cdot 7H_2O$, but only 5 per cent NaCl. At 15 per cent $MgSO_4 \cdot 7H_2O$ or at 3 per cent NaCl, the colonies grew well.

Stapp has also shown that actinomycetes can tolerate a high salt concentration. Many strains were able to grow in a 10 per cent concentration of KNO_3; NaCl was tolerated by some strains in a maximum 8 per cent concentration, 6 per cent by others, and only 1 per cent by still others; some strains tolerated 30 per cent $NaSO_4$ and others only 2 per cent. Some strains were able to grow in a 10 per cent concentration of $MgSO_4$, and one grew weakly even in a 50 per cent concentration of the salt.

Sodium thiosulfate was tolerated in 10 per cent concentration. KI and KBr were tolerated in 5 per cent concentration; LiCl only up to 0.5 per cent by one strain and CsCl up to 0.1 per cent. $SrCl_2$ permitted the growth of some strains in 1 per cent concentration.

Some strains are resistant to salts usually considered as toxic.

In earlier studies on the growth of actinomycetes, Neukirch (1902) observed that *S. ochroleucus* grew in broth containing 2 per cent NaCl. Lachner-Sandoval (1898) reported that good development of *S. albidoflavus* took place in a medium containing 16 per cent NaCl. The fact that actinomycetes are found in salt-rich substrates, such as curative muds (Rubentschik) and in the sea close to shore, speaks for their adaptation to salt-rich environments. Krassilnikov reported on the ability of various actinomycetes (*Streptomyces*, *Nocardia*) to grow well in the presence of 10 per cent NaCl or 20 per cent Na_2SO_4. He noted that the majority of actinomycetes are able to grow in higher salt concentrations than bacteria. This was true particularly of the pigmented organisms.

These results tend to suggest that the role of metallic elements as integral parts of cell systems and of catalytic processes of enzymes is of considerable importance. McElroy and Nason presented certain patterns which appear to be evolving from physicochemical and nutritional studies of metals in various metabolic reactions. Molybdenum, copper, and iron are said to be most closely associated with electron-transferring systems. These metals are not required specifically for the combination of substrate to protein, but rather function as "electron couplers" from one protein system to another. Magnesium and, to some degree, manganese function primarily in group transfer reactions, particularly those involving phosphate. Manganese and, to a lesser degree, zinc and magnesium, predominate in general enzymatic decarboxylation and hydrolysis reactions.

Some metals form a stable metal-protein complex and in such cases are considered to be specific. In other cases, there are enzymes in which the metal may easily be separated from the protein and be replaced in function by a metal of equivalent valency.

Metals may also play an important role as a structural part of a specific molecule. Examples of this kind are the iron in the antibiotic grisein, cobalt in vitamin B_{12}, and iron in a streptomycete pigment known as ferroverdin.

Outward manifestations of the effects of metals upon a microorganism can be observed as changes in growth, sporulation, and ability to utilize certain substrates as well as the formation of metabolic products such as pigments, antibiotics, and vitamins. The effect of metals on streptomycete nutrition has been studied largely from the point of view of antibiotic production. It is common practice to use complex organic media to which are added mineral salt solutions containing the metals considered to be beneficial for growth and antibiotic formation. The metals usually used are potassium, magnesium, iron, zinc, copper, calcium, and manganese. Streptomycin formation by *S. griseus* has been studied more than any other reaction in connection with the effects of metals in the actinomycete system.

CHAPTER 9

Biochemical Activities

The manifold aspects of the biochemistry of actinomycetes are still far from elucidated. Nevertheless, considerable progress has been made in recent years in our understanding of some of the chemical mechanisms involved in the growth and activities of these organisms. By far the greatest number of investigations on the activities of actinomycetes have been concerned largely with antibiotic production and the mode of action of antibiotics. Limited consideration has also been given to certain other fundamental principles of actinomycete biochemistry.

Respiration

Hockenhull *et al.* reported that, under highly aerobic conditions, glucose was converted by *S. griseus* mainly to cell material and CO_2. Under restricted aeration, lactic acid was also formed. Pyruvic acid was produced during the stages of most rapid growth. The metabolism of glucose by actinomycetes was dependent upon the presence of phosphate, the optimal hydrogen-ion concentration for both glucose oxidation and the rate of disappearance of inorganic phosphate being about pH 7. At pH 7.3, there was a gradual increase in glucose utilization from 0.62 mg/ml, in the absence of phosphate, to 1.64 mg/ml, in presence of 0.01 M potassium phosphate. Further increases in phosphate concentration did not affect the utilization of the sugar.

Phosphate esters, tentatively identified as glucose 1-phosphate and glucose 6-phosphate, were obtained by Hockenhull in fluoride-inhibited systems. Glucose oxidation was depressed by 10^{-3} M sodium iodoacetate and by 10^{-2} M sodium arsenite, but was stimulated by 10^{-2} M sodium arsenate; 10^{-3} M 2,4-dinitrophenol and 10^{-3} M sodium azide had no effect. Streptomycin production was decreased by 3×10^{-3} M sodium arsenate but not by 10^{-2} M sodium fluoride or 10^{-2} M sodium iodoacetate. *S. griseus* metabolized members of the tricarboxylic acid cycle, although citrate and α-ketoglutarate gave much lower values of CO_2 at pH 7.3 than did pyruvate, acetate, succinate, fumarate, or malate. Keto acids were produced, in presence of arsenite, from fumarate, malate, glucose, lactate, acetate, succinate, glutamate, and citrate in descending order of yield. Except from fumarate, which yielded some material behaving like α-ketoglutarate, the product was chiefly pyruvate.

According to Cochrane and Hawley, ribose 5-phosphate is oxidized by extracts of *S. coelicolor* almost to completion, in a series of reactions dependent on triphosphopyridine nucleotide, stimulated by thiamine pyrophosphate, and insensitive to iodoacetate. Fructose 1,6-diphosphate is dephosphorylated by one or more enzymes, with optimal activity at pH 4.5 to 5.0; it also can be oxidized by triphosphopyridine nucleotide-dependent reactions, involving glucose 6-phosphate as an intermediate. The conclusion was reached that *S. coelicolor* can metabolize glucose aerobically through either or both of two pathways, the "hexosemonophosphate" and the Embden-Meyerhof system.

According to Douglas and San Clemente, homogenized suspensions of *S. scabies* grown under submerged aerated conditions are able to oxidize glucose, succinate, fumarate, citrate, and acetate when placed in a Warburg-type manometric respirometer. The high endogenous respiration is due to oxidation of nitrogenous substances; the cells also oxidize glutamate, aspartate, threonine, phenylalanine, glycine, and valine and leucine to some extent. It was suggested that tyrosine is oxidized through dihydroxyphenylalanine with subsequent formation of melanin. Addition of dinitrophenol increases the oxygen uptake by cells in contact with pyruvate and α-ketoglutarate, and decreases the uptake in cells metabolizing glucose.

As pointed out previously, anaerobic actinomyces cultures are able to fix CO_2. This has been confirmed by Pine, using C^{14}, as brought out in Table 31. Succinic acid produced by this organism had a high percentage of its radioactivity in the methylene carbons: "When grown in the presence of oxygen, 90 per cent of the total activity of the succinic acid was found in the methylene carbons as compared to 62 per cent when it was grown anaerobically."

Butterworth *et al.* (1955) have shown that a C_3-C_1 condensation coupled with tricarboxylic acid cycle activity is a major pathway of carbon dioxide fixation by *S. griseus*. The intramolecular distribution of C^{14} in glutamic acid, produced by the incorporation of labeled carbon dioxide, confirmed the theory that the tricarboxylic acid cycle operates extensively in the terminal respiration of *S. griseus.*

Gilmour *et al.* (1955) demonstrated the oxidation of tricarboxylic acid cycle intermediates by nonproliferating cells of *S. griseus.* Succinate and α-ketoglutarate were oxidized only at substrate concentrations higher than those normally employed. The cells had to be preincubated in order to obtain oxidation of fumarate, malate, and citrate. Gilmour *et al.* have further shown that the labeling pattern suggests that glutamic acid arises from acetate through extensive operation of the conventional tricarboxylic acid cycle. The role of this acid cycle in amino acid synthesis is established by the incorporation of radioactivity into most of the amino acids of the cellular protein from proliferating cells utilizing acetate-1-C^{14}.

According to Perlman, most of the studies on the metabolism of carbohydrates by *S. griseus* tended to indicate that carbon dioxide is the main metabolic product; no accumulation of significant quantities of intermediates in this oxidation has been reported. Some doubt was expressed whether the Krebs tricarboxylic acid cycle operates in *S. griseus* metabolism of glucose (Garner and Koffler). The report of succinic acid production by other species suggested that at least some of the carbohydrate is metabolized by these organisms by way of a carboxylic acid cycle (Cochrane *et al.*). Lactic

TABLE 31

Anaerobic fermentation of glucose plus carbon dioxide-C^{14} by Streptomyces *sp.* (Pine)

Substrate or product	μmoles/ml	cpm/μmole
Glucose utilized	34.4	
Carbon dioxide (initial)	33.4	523.4
Carbon dioxide (final)	14.1	492.8
Formic acid	12.6	50.6
Acetic acid	10.3	.36
—COOH		.23
—CH_3		.13
Lactic acid	43.2	
Succinic acid	11.0	527.2
—COOH		143.3
—CH_2		120.3
Beta alanine (predicted)		383.9
(found)		377.0

acid has been reported in *S. griseus* and in certain *S. lavendulae* fermentations, and acetic acid in others. These acids have been looked upon as transitory products accumulating under fermentation conditions and inhibiting normal growth of the actinomycete. They are further metabolized by these organisms if the fermentation period is extended or if aeration is increased. In certain *S. fradiae* cultures where the medium is poorly buffered, sufficient acetic acid accumulates to inhibit further growth and metabolism (Hubbard and Thornberry). The accumulated acetate is metabolized when the pH of the medium is raised.

Wang *et al.* (1958) carried out time course experiments on the oxidation of C^{14}-labeled glucose and acetate by *S. griseus.* With

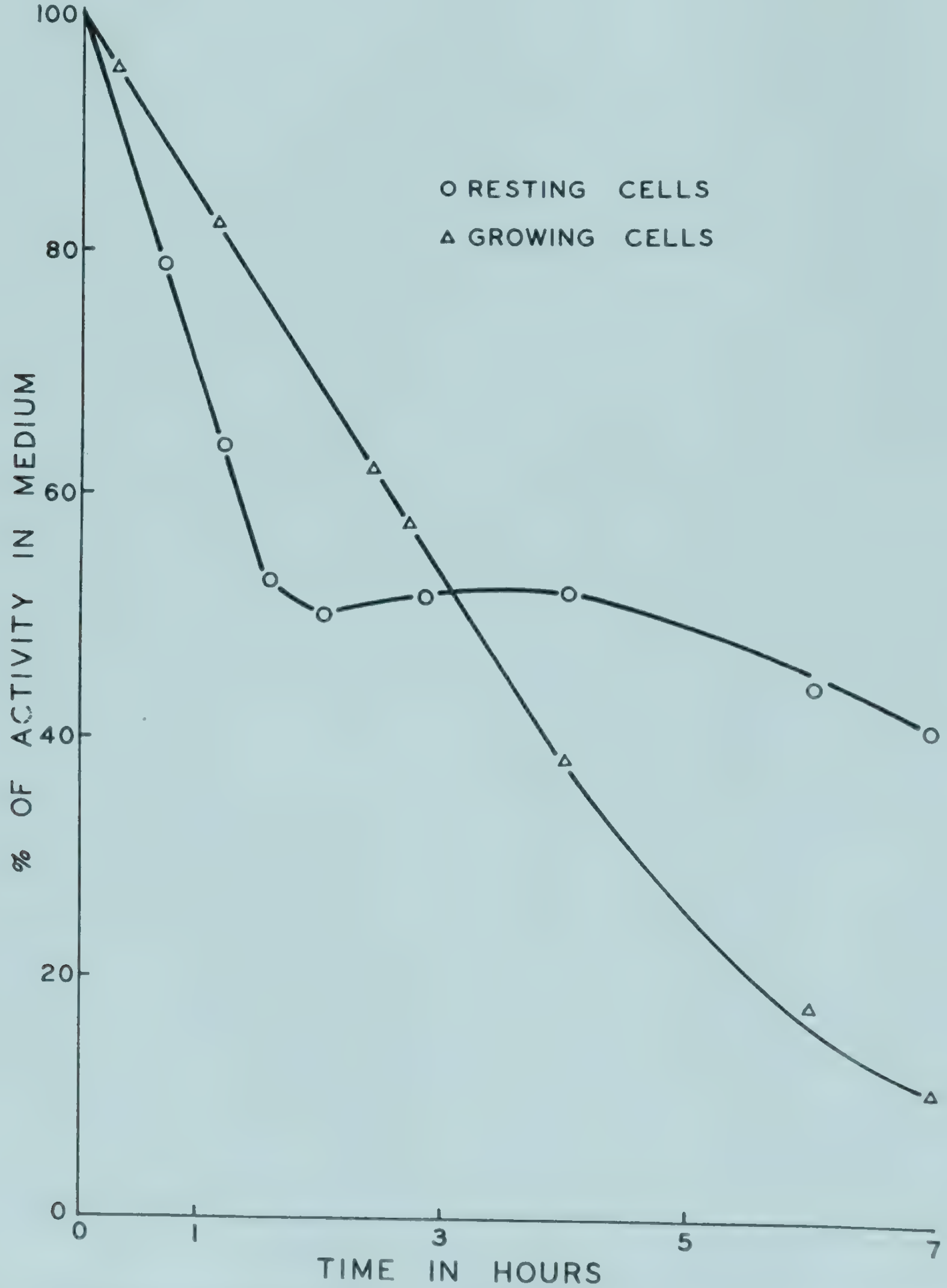


FIGURE 70. Utilization of acetate-1-C^{14} by growing and resting cells of *S. griseus* (Reproduced from: Gilmour, C. M. *et al.* J. Bacteriol. 69: 721, 1955).

young cells, considerably more glucose-C^{14} activity was detected in cellular constituents than with older cells. Much larger quantities of fermentation products were found in the medium in the case of the latter, however. With respiratory $C^{14}O_2$, there was a decrease in the individual chemical recoveries with an increase in the age of the culture. The Embden-Meyerhof-Parnas glycolytic scheme was believed to be responsible for the major portion of glucose breakdown, only a minor part going via the phosphogluconate decarboxylation route. The age of the cells may influence, however, the extent of utilization of either pathway but not alter the over-all pattern of glucose metabolism.

Propionic acid has been reported as a metabolite in micromonospora fermentation (Hungate). Media containing a large number of carbohydrates will support the growth of *S. griseus*; streptomycin production, however, is low or absent unless glucose, starch, or maltose is present (Dulaney and Perlman). Streptomycin containing C^{14} has been obtained when *S. griseus* was grown on media containing carbohydrate labeled with C^{14}. Addition of inorganic phosphate to *S. griseus* fermentations results in increased rates of glucose utilization. It results in an almost complete suppression of streptomycin production.

Further studies on respiration of *S. griseus* have been made by Gottlieb and Anderson, Oginsky *et al.*, and Inoue, whose work was reported in Chapter 7.

Extensive studies on the respiration of various carbohydrates by various nocardias were made by McClung *et al.* (1958). Warburg manometric techniques and cell suspensions in which endogenous respiration was minimal (twice washed cells shaken overnight in pH 7.0 buffer) were used. The efficiency of carbon utilization was: glucose, fructose > sucrose, mannose, galactose > maltose > sorbose > lactose > arabinose > cellobiose > rhamnose. When one of the less readily utilized sugars was replaced in part by glucose, the oxygen uptake was considerably higher than with either sugar alone, showing that the glucose stimulated the utilization of the other sugar. Growth patterns paralleled the respiration patterns.

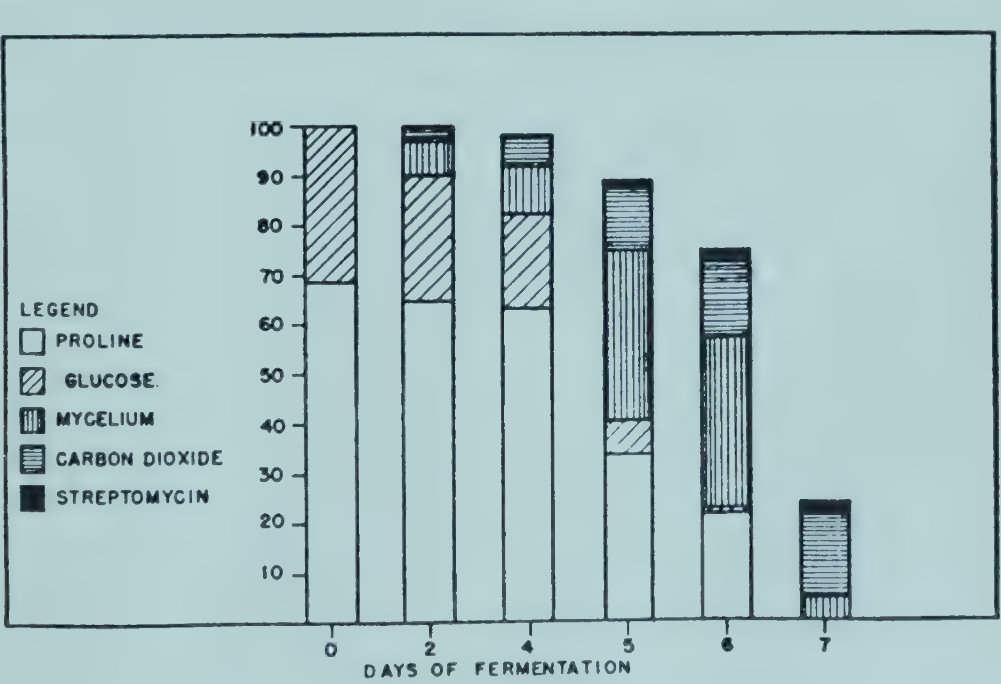


FIGURE 71. Carbon balance of *S. griseus* fermentation (Reproduced from: Woodruff, H. B. and Ruger, M. J. Bacteriol. 56: 318, 1948).

Oxidation of Steroids and Related Compounds

It has long been recognized that actinomycetes are capable of decomposing fats (Netchaieva). Until very recently, it was commonly assumed that the only actinomycetes capable of oxidizing steroids are found largely among the species of *Nocardia*. Turfitt, for example, demonstrated by means of enrichment culture methods, that the breakdown of cholesterol and other steroids in soils is carried out primarily by nocardias, especially *N. erythropolis*. Among the other active species, he listed *N. aquosa*, *N. globerula*, *N. coeliaca*, and *N. restricta*.

More recently, however, different streptomyces (*S. griseus*, *S. fradiae*, *S. venezuelae*, and *S. aureofaciens*) were found (Sisler and Zobell, Perlman *et al.*, Hirsch *et al.*, Herzog *et al.*) capable of oxidizing steroids and lipids. Some species oxidized lipids completely to carbon dioxide, without the formation of any intermediate metabolites.

Washed cells of *S. albus* are able to convert estradiol to estrone (Welsch and Hensghem); pregnenolone is converted to progesterone by *S. griseus*, and progesterone to hydroxy-progesterone by various actinomycetes (Perlman *et al.*). The conversion of progesterone-(Δ^4-pregnene-3,20-dione) to $\Delta^{1,4}$-androstadiene-3,17-dione by Fried *et al.* suggested that the enzymes which carry out this transformation are adaptive in origin. The metabolism of progesterone by washed *S. lavendulae* cells grown in media supplemented with progesterone or in unsupplemented media was inhibited by azide, cyanide, or arsenite but not by selenite or fluoride (Perlman *et al.* 1953). The conversion of progesterone by cells grown in unsupplemented media was also inhibited by the addition to the progesterone-cell-suspension of certain antibiotics, such as streptomycin. Addition of any of the antibiotics to the steroid-cell-suspension 12 or more hours after the steroid was mixed with the washed cells had no effect on the conversion. The antibiotics had no effect on the progesterone-oxidizing ability of washed cell suspensions of *S. lavendulae* grown in media supplemented with progesterone.

According to Vischer *et al.*, submerged cultures of various streptomyces are able to convert cortexone to 16α-hydroxycortexone.

Saburi *et al.* isolated several streptomyces which utilize cholic acid as the sole source of carbon. The utilization of these acids depends on the nuclear constitution and the length of the side chains of the bile acids. *S. gelaticus* was found capable of converting cholic acid in a C-22 acid ($C_{22}H_{28}O_4$). When *S. gelaticus* was cultured in a synthetic medium containing cholic acid as the sole source of carbon, it oxidized cholic acid to an acid with melting point 280 to 282° (decomposes), which was presumed to be 7α-hydroxy-3,12-dioxo-Δ^4-bisnorcholenic acid. When it was grown in a medium containing cholic acid and glucose as the carbon sources, various intermediates such as $7\alpha,12\alpha$-dihydroxy-3-oxocholanic acid, 7α-hydroxy-3,12-dioxocholanic acid, 7α-hydroxy-3,12-dioxo-Δ^4-cholenic acid were formed (Hayakawa *et al.*). Hayakawa *et al.* (1958) later demonstrated that *S. rubescens* possesses an alternate pathway for the degradation of cholic acid from that of *S. gelaticus*.

According to Collingsworth *et al.*, *S. fradiae* is able to convert 11-desoxy-17-hydroxycortisone (comp. S) to 17-hydroxycorticosterone (comp. F).

Webley and deKock studied the oxygen uptake by washed suspensions of *Nocardia opaca*. The uptake was increased by the presence of *n*-dodecane, *n*-tetradecane, *n*-hexadecane, *n*-octadecane, and paraffin wax. Decyl, lauryl (dodecyl), and octadecyl alcohols also gave increased oxygen uptake, but amyl, *iso*amyl, *iso*hexyl, and heptyl alcohols were toxic. The long-chain fatty acids (C_7–C_{16}) were all metabolized at very low concentrations (0.0012 *M*).

According to Webley *et al.* (1955), the mechanism of breakdown of ω-phenyl-substituted fatty acids by *N. opaca* was as follows: Acids with an odd number of carbon atoms in the side chain (phenylpropionic, phenylvaleric, and phenylheptylic acids) were converted to benzoic acid, and cinnamic acid was an intermediate; o-hydroxyphenylacetic acid was identified as a common product when acids with an even number of carbon atoms (phenylacetic, phenylbutyrix, phenylcaproic, and phenylcaprylic) were used, thus supporting the theory of β-oxidation as a mechanism of breakdown of short-chain fatty acids by *N. opaca.* Webley *et al.* (1957) later demonstrated that a strain of *N. opaca* will convert 3- and 4-monochlorophenoxybutyric acids to the corresponding substituted acetic acids. During these conversions an intermediate is formed which proved to be β-hydroxy-γ-(4-chlorophenoxy) butyric acid.

Adelson *et al.* found in soil a strain of *S. griseus* that had the capacity to bring about demyelination of bovine spinal cord *in vitro.* Just what mechanisms this process involves are still difficult to tell.

Proteolytic Activities of Actinomycetes

Actinomycetes are capable of attacking a large number of plant and animal proteins, as pointed out elsewhere. Macé was among the first to demonstrate their marked proteolytic properties. The proteins are hydrolyzed to amino acids, polypeptides, and ammonia. Waksman and Starkey compared the utilization of proteins of animal and plant origin by bacteria, fungi, and actinomycetes. The actinomycetes were found to occupy an intermediate position between the other two groups of organisms in the ratio of protein decomposed to protein synthesized. The presence of a carbohydrate was found to be of great importance in influencing both the amount of protein decomposed and that of cell material synthesized. When proteins were used as a source of both carbon and nitrogen, the reaction of the medium changed rapidly to alkaline. Considerable ammonia, liberated in the decomposition of the proteins, was lost by volatilization.

A number of amino acids can be utilized by actinomycetes in synthetic media. This is true of proline, glutamic acid, arginine, aspartic acid, and histidine. The fact that actinomycetes can utilize ammonia and nitrate as sources of nitrogen, and that amino acids are degraded to ammonia, suggests that the carbon residue of the amino acids may be more important in the actinomycete economy than are the nitrogen-containing groups. Problems of deamination and amino acid degradation by streptomycetes have been discussed by Gottlieb and Ciferri.

Actinomycetes are characterized by intense activity in breaking down proteins to ammonia. This is true especially when a long period of incubation is employed. These organisms are capable of allowing a large accumulation of ammonia even in the presence of available carbohydrates.

When washed cell material of *S. lavendulae* was shaken with different amino acids at pH 6.8, deamination could be measured by ammonia formation. Arginine and histidine were acted upon most readily; β-alanine was deaminated only about one-third as readily as *D*-alanine; leucine, isoleucine, and certain other amino acids were deaminated not at all or only in mere traces.

The formation of proteolytic enzymes are discussed in detail in Chapter 11.

Decomposition of Keratins

Actinomycetes possess the unique capacity to decompose keratins. This was shown first in connection with the ability of certain pathogenic forms to attack the skin and horny portions of feet (Acton and McGuire). When a soil is enriched with keratinized tissues, such as human hair and feathers, organisms belonging to the genus *Actinoplanes*

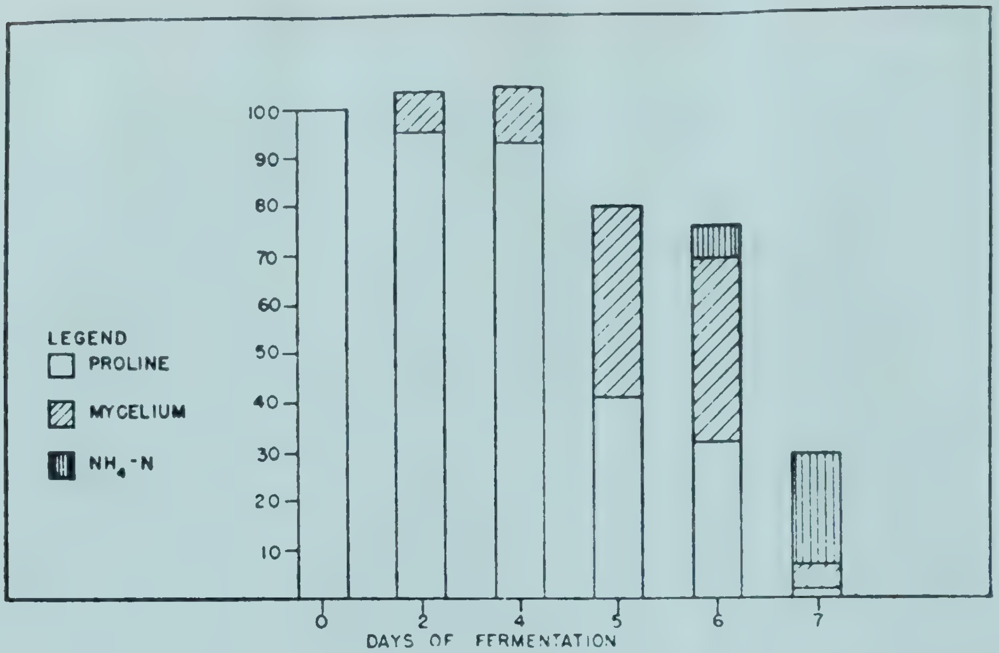


FIGURE 72. Nitrogen balance of *S. griseus* fermentation (Reproduced from: Woodruff, H. B. and Ruger, M. J. Bacteriol. **56:** 319, 1948).

TABLE 32

Ability of various species of actinomycetes to digest wool (Noval)

Species	Wool digested after various incubation periods, in per cent			
	6 days	7 days	8 days	30 days
S. fradiae 3739	10	20	70	90
S. fradiae 3535	0	10	50	90
S. fradiae 3572	0	0	20	90
S. fradiae 3719	0	0	0	90
S. rimosus	0	0	0	30
S. griseus 3475	0	0	0	10
S. griseus 3464	0	0	0	30
S. griseus 3492	0	0	0	30
S. roseochromogenes	0	0	0	10
S. aureus	0	0	0	0
S. albus	0	0	0	0
N. polychromogenes	0	0	0	40

will develop (Karling, Gaertner, Rothwell). The mechanism of keratin destruction by these organisms has not been determined.

Jensen (1930) added to moist soil, keratin prepared from horn meal and allowed it to decompose. The process of keratin decomposition was slow; it led to a steady accumulation of ammonia and nitrate in the soil. After 120 days, 35 to 40 per cent of its nitrogen was transformed into nitrate. The addition of keratin produced little or no increase in the number of bacterial colonies on agar platings, but markedly increased the number of actinomycete colonies. Two actinomycete strains were isolated and found capable of thriving on keratin in pure culture; they decomposed this substance with the formation of ammonia. One of the strains could be recognized as *S. citreus*. The other strain was not named, but corresponded closely to the description of Waksman's *Streptomyces* 145. The presence of keratinolytic actinomycetes belonging to the genus *Streptomyces* in the soil has been demonstrated by various other investigators (Piechowska, Hirschmann *et al.*).

Detailed studies of the decomposition of hoof meal, horn meal, leathers, and similar materials were made by Noval and Nickerson and Noval (Table 32). These investigators succeeded in isolating an enzyme preparation (keratinase) from a culture of *S. fradiae*, as is shown in Chapter 11.

Decomposition of Chitins

Chitin-decomposing actinomycetes are widely distributed in nature. As many as 85 per cent of the *S. alboflavus* group, 84 per cent of the *S. albus*, 83 per cent of the *S. rubrireticuli*, 82 per cent of the *S. griseus*, 75 per cent of *S. scabies*, and only a few (36 per cent) of the *S. violaceus* groups possess this capacity. Only a few types of chitin-decomposing actinomycetes have been found in forest soils. This may be due possibly to the lack of small arthropoda in such soils.

The actinomycetes attacking chitins do so by means of certain enzymes, designated as chitinase. Berger and Reynolds studied these enzyme systems found in the culture filtrates of a streptomyces capable of hydrolyzing chitin. The following compounds were formed during the digestion process: *N*-acetylglucosamine, a β-1,4-linked disaccharide, N,N′-diacetylchitobiose. At least two enzymes were found in the mixture: 1, a heat-labile fraction was able to split the disaccharide to glucosamine, but was unable to hydrolyze the chitin; 2, a heat-stable fraction contained a chitinase which hydrolyzed the chitin to equimolar concentrations of the two fractions, but did not cleave the disaccharide; the system is specific for polymers of N-acetylglucosamine. The chitobiase hydrolyzed the β-phenyl glucoside of N-acetylglucosamine but not the glucosides of glucosamine or glucose. Further information on chitinase is given in Chapter 11.

Decomposition of Cellulose

As pointed out previously, various actinomycetes inhabiting soils, high-temperature composts, and sewage sludge are capable of attacking cellulose. Krainsky, Waksman, and Brussoff were among the first to demonstrate the capacity of various actinomycetes to carry out this process. Unfortunately, little is known of the enzymatic systems involved in the action of these organisms upon cellulose. The only products obtained are usually slimy materials and pigments that range from red and yellow to blue and black. Further information on cellulase is given in Chapter 11.

In a study of cellulose decomposition by termites, Hungate isolated a culture of a micromonospora that decomposed cellulose under anaerobic conditions. The presence of complex organic substances in the medium is required. Among the products formed, acetic and propionic acids were identified, in addition to CO_2. An old culture of micromonospora contained cellulase that converted the cellulose to glucose. Hungate isolated another strain of the anaerobic micromonospora from a culture of protozoa from the rumen of cattle. The organism is referred to as *M. propionici*.

Decomposition of Starches and Hemicelluloses

Starches are also decomposed by numerous actinomycetes. The problem of isolating diastatic enzymes is much simpler, since it is very easy to study this process and isolate final products.

Hemicelluloses and polyuronides, including mannans, galactans, glucans, xylans, as well as pectins, agar (Stanier), and others, are decomposed by a large number of actinomycetes. Some of these actinomycetes are more active than fungi.

Further information on amylase and cytase is given in Chapter 11.

Decomposition of Rubber

Söhngen and Fol (1914) reported that actinomycetes are capable of attacking rubber, bringing about a reduction in viscosity and transformation into CO_2. Several species, notably *A. fuscus* and *A. elastica*, were isolated. They were found capable of utilizing various salts of organic acids, including stearate and palmitate, but not formate.

Kalininko came to the conclusion that the decomposition of rubber in nature is carried out by molds and, especially, by actinomycetes. Among the latter organisms, three forms were found to be particularly active, namely, *S. coelicolor*, *S. aurantiacus*, and *S. longisporus ruber*. Further studies on rubber decomposition have been made by Spence and van Niel.

Decomposition of Paraffin Hydrocarbons

Büttner established that various aerobic actinomycetes that appear to include species of both *Streptomyces* and *Nocardia* are capable of attacking paraffin. A film is formed upon the paraffin. Most of the lost paraffin is recovered as CO_2. Oxidation of petroleum hydrocarbons by marine organisms was studied by Zobell *et al.* See also Chapter 7, for hydrocarbon oxidation by nocardias.

Production of Odors

Many actinomycetes, especially species of *Streptomyces*, are characterized by the production of a specific odor, which is typical of freshly plowed soil. It is musty, or earthy, and occasionally fruity in nature. Rullmann believed that the odor is characteristic of certain species. According to Lieske, only those aerobic forms that produce chalky white aerial mycelium with round spores are capable of forming this odor; the nonsporulating forms of the *Nocardia* type and those streptomyces that produce cylindrical spores do not give rise to any odor. The presence of carbohydrates in the medium favors odor production. The thermophilic actinomycetes are responsible for the more fruity scents, which arise particularly from young cultures. The odoriferous substance can be extracted from the culture. It is soluble in ether and partly in alcohol.

Thaysen (1935) found that the odor is produced by certain actinomycetes only under certain conditions of growth and on certain media. Gelatin, for example, does not favor the formation of odoriferous substances.

As is shown in Chapter 3, the odor produced by actinomycetes is responsible for a certain type of spoilage of fish that absorb it into their digestive systems, from which it spreads throughout the bodies of the fish.

Lime Precipitation

Nadson isolated various actinomycetes from the bottom of a lake characterized by limestone precipitation. He mentioned *A. albus*, *A. roseolus*, and *A. verrucosus*, all apparently belonging to the genus *Streptomyces*. He considered them as members of the microbiological population active as geological agents. Molisch (1925) also observed cultures of actinomycetes capable of bringing about lime precipitation. Colonies growing on media containing Ca acetate, 2 per cent solution, are surrounded by crystals of calcium carbonate.

Krassilnikov (Issatchenko, 1948) observed the precipitation of $CaCO_3$ on roots of plants covered with actinomycetes. In two old cultures of actinomycetes isolated from salt lakes, Rubentschik (1948) observed the crystallization, in protein media, of $CaCO_3$.

Nitrification and Nitrate Reduction

Various reports have been made (Schatz *et al.*) that certain actinomycetes are able to oxidize ammonia and nitrite to nitrate. Jensen (1951) reported that *N. corallina* can nitrify up to 64 per cent of oxime of pyruvic acid in an inorganic salt solution. Glucose inhibited nitrification, by stimulating synthesis of cell material, at a C:N ratio above 20:1. In peptone media, *Nocardia* converted the oxime almost quantitatively to nitrite, the rate of its formation being equal to that of cell synthesis.

The reduction of nitrate to nitrite by actinomycetes has long been recognized (Salzmann, Joshi, Ghosh *et al.*). Various organisms differ, both qualitatively and quantitatively, in this connection. Fedorov and Kudriasheva (1955) found in cultures of actinomycetes with nitrate as a source of nitrogen, traces of nitrite and hydroxylamine in far lower concentrations than the amount of nitrate that disappeared. They reached the conclusion that the reduction took place to molecular nitrogen. The reaction of the medium remained acid, presumably because of the formation of organic acids from the sugar (Table 33).

TABLE 33

Reduction of nitrates by actinomycetes under anaerobic conditions, in a nitrogen atmosphere
(Fedorov and Kudriasheva)

Organism	Glucose consumed, mg	Mycelium produced, mg	Nitrogen content of mycelium, mg	NO_3-N disappeared, mg	NO_3-N loss, mg
S. globosus	488	8.6	1.12	261	253
	146	9.2	1.06	171	159
S. globisporus vulgaris	110	4.6	0.50	195	191
	104	3.4	0.36	171	168
S. globisporus x	178	10.0	0.15	165	165

Nitrogen Fixation

Various reports have been made in the past of the ability of one or more actinomycetes to fix atmospheric nitrogen. Fedorov and Kudriasheva (1956) came to the conclusion that various actinomycetes are capable of fixing atmospheric nitrogen, thus confirming the claims of Emerson, Carter and Greaves (1928), Kober (1929) and von Plotho (1940). On the other hand, such claims were denied by Beijerinck (1900), Fousek (1912), Münter (1916), Waksman (1920), and Krassilnikov (1938). Repeated studies by Waksman (1920), Allison *et al.* (1934), and Shinobu (1953) failed to confirm the reports.

Recent evidence seems to suggest a nitrogen-fixing capacity for certain particular species, as reported for *A. spinae* by Velich. Metcalfe and Brown (1957) described two nocardias, *N. calcarea* and *N. cellulans*, isolated from grassland lime soils. These cultures were found to have the capacity to fix atmospheric nitrogen to the extent of 2.0 to 4.5 mg of N/gm of glucose or other carbon source in the medium. The second culture was also capable of decomposing cellulose, the amount of nitrogen fixed being 5 to 12 mg of N/gm of cellulose decomposed.

Acid Production

As pointed out previously, during the growth of actinomycetes in media containing proteins, amino acids, or nitrates as sources of nitrogen, the reaction tends to become alkaline, even in the presence of sugars as sources of carbon; when ammonium salts are present as the sole sources of nitrogen, the reaction may become acid. Certain actinomycetes, however, are capable of forming from carbohydrates various organic acids, depending on the nature and concentration of the carbohydrate. It has been suggested that the Krebs tricarboxylic acid cycle operates in the metabolism of glucose by at least certain species of actinomycetes, as in the case of *S. griseus* (Inoue).

S. lavendulae gives rise to fairly large amounts of lactic acid (Table 34), provided sufficient carbohydrate is present in the medium; the pH changes to 5.7 in the presence of 5 per cent glucose (some actinomycetes produce enough acid to reduce the pH as low as 4.6). Lactic acid is not found in any

TABLE 34

Acid formation from glucose by S. lavendulae
(Woodruff and Foster)

Glucose concentration, per cent	pH of medium after			
	3 days	4 days	5 days	6 days
0	8.2	8.6	8.7	8.8
1	6.8	6.9	7.0	7.4
2	6.5	6.5	6.5	6.5
5	6.2	6.1	5.7	5.7

large concentrations in the cultures of the sporulating strains of *S. griseus*, but the aerial mycelium-free mutants are capable of forming considerable amounts of this acid. Lactic acid also appears to be a characteristic metabolic product of various nocardias, as shown by von Plotho and others.

The formation of succinic acid by various species indicates that at least some of the carbohydrate is metabolized through the carboxylic acid cycle. During the growth of *S. fradiae* in certain poorly buffered media, sufficient acetic acid accumulates to inhibit further growth and metabolism of the organism. In these cases, the accumulated acetate is metabolized when the pH of the medium is raised. Propionic acid has been reported as a metabolite in the growth of micromonospora.

Cochrane and Dimmick found that, in a glucose medium in the presence of an excess of $CaCO_3$, *S. coelicolor* produced large amounts of succinic acid, small amounts of lactic acid, and traces of fumaric and an unidentified keto acid. Volatile acids were not detected. This property of forming acids from sugar was believed to be characteristic of the particular species.

Numerous other investigators reported that actinomycetes are able to produce organic acids from carbohydrates. Magnus observed that many of the actinomycetes found in the larynx are able to form lactic type acids even in sugar-free media. von Plotho confirmed these observations. Woodruff and Foster demonstrated that *S. lavendulae* is capable of producing considerable amounts of lactic acid from carbohydrates. The nature of the nitrogen source and the concentration of sugar are of considerable importance in this connection. In the presence of glycine, more sugar was consumed and less lactic acid produced than with tryptone as a source of nitrogen. Without glucose or with low concentrations in the tryptone medium, ammonia accumulated. With 1 per cent glucose, the pH was lowered appreciably, even in buffered media, as a result of the formation of organic acids; with 2 per cent glucose, especially in unbuffered media, the pH levels went down to as low as 3.2 in 2 days.

Actinomycetes are capable of utilizing readily various organic acids. Some of the higher molecular acids are converted in the process to lower molecular acids. Martin and Batt have shown that the oxidation of propionic acid by cell suspensions of *Nocardia corallina* is stimulated by carbon dioxide. Thiamine-deficient cells convert propionate to pyruvate with a simultaneous accumulation of succinic acid. Two oxidation pathways to pyruvate were demonstrated; only one of these included a symmetrical intermediate (probably succinate).

According to Masuo and Kondo, various streptomyces accumulate α-ketoglutaric acid, with or without some pyruvic acid from glucose or glycerol. Some organisms convert glycerol to dihydroxyacetone, and D-sorbitol to L-sorbose.

Among the other metabolic products of actinomycetes, it is sufficient to mention the lactone of β-hydroxy-α,α',γ-trimethyl pimelic acid, a substance found as a degradation product of certain antibiotics (Anliker *et al.*).

Chemical Composition of Actinomycetes

Most of the studies of the chemical composition of the spores and mycelium of cultures of actinomycetes grown on different media have been limited to the elementary composition of the dry cell material. In a few cases, the organic chemical constituents have been examined. The composition of the medium in which the cultures are grown is of considerable importance in this connection.

Stokes and Gunness concluded that the amino acid content of an organism is, qualitatively and quantitatively, a stable and characteristic property of the cell under fixed conditions of growth. Addition of glucose to the medium in which *S. griseus* was grown reduced the concentration of some of the amino acids (arginine, histidine, lysine), but not of others (tryptophan). The nitrogen content of the culture was about 10.5 per cent, independent of addition of sugar.

Witter (1933) was unable to find either chitin or a definite nucleus in the species of actinomycetes examined, a characteristic that distinguishes them definitely from the true fungi (See also Schmidt). Hagedorn established an isoelectric point for actinomycetes also similar to that of bacteria.

Detailed cell wall studies support the bacterial nature of the actinomycetes. No evidence was found of the presence of polymers, such as chitin, mannan, glucan, or cellulose, which are found in yeasts and true fungi. The cell wall composition in all of the actinomycetes studies resembled the cell wall composition of gram-positive bacteria. The majority of the streptomycetes are lysed by lysozyme; this property is characteristic of many gram-positive bacteria and has been shown to be due to lysis of the cell wall.

Avery and Blank (1956) could not find any chitin or cellulose in representative cultures belonging to the genera *Actinomyces*, *Nocardia*, *Streptomyces*, and *Micromonospora*. They concluded on the basis of these and other data that these organisms belong to the bacteria rather than the fungi. An analysis of a polysaccharide isolated from cultures of *Nocardia asteroides* contained arabinose and galactose in a molar ratio of 1.7:1. By isolation in crystalline form, the two monosaccharides were identified as D-arabinose and D-galactose. Partial hydrolysis showed that some of the D-arabinose units in the polysaccharide were in the furanoside ring whereas the D-galactose units possessed the pyranoside structure. Methylation studies showed that the polysaccharide was a branched structure of D-arabinose and D-galactose units with some of the arabinose forming nonreducing, terminal residues. This evidence points further to the close taxonomic relationship between *N. asteroides* and *Mycobacterium tuberculosis* (Bishop and Blank).

According to Guinand *et al.* (1958), the mycelium of *N. asteroides* yielded on extraction with 1:1 alcohol-ether and chloroform, a mixture of lipoproteids, consisting partly of peptides containing six amino acids and partly of acid lipids.

Romano and Nickerson made a study of the cell wall of *S. fradiae*. The cells were first broken in the Mickle disintegrator; the cell walls were then readily lysed by lysozyme. On hydrolysis with 2 *N* hydrochloric acid, reducing substances, accounted for largely by hexosamine, were liberated. The cell wall, like that of gram-positive bacteria, appears to contain a mucopolysaccharide in association with protein.

According to Sohler and Romano, the cell wall of *Streptomyces* species is mucoid in nature, is lysed by lysozyme, and contains considerable amounts of hexosamine (Table 35). On the other hand, the cell wall of *Nocardia* is not susceptible to the action of lysozyme, contains much smaller amounts of hexosamine, but contains 10 per cent pentose, tentatively identified as arabinose.

Further results on the chemical composition of the cell walls of actinomycetes were reported by Sohler *et al.* (1957). The action of lysozyme upon the mycelium is limited primarily to the cell wall, since isolated cell walls were completely lysed. The cell wall of *S. fradiae* was found to be composed of a mucopolysaccharide of which the major carbohydrate constituent is glucosamine. This was demonstrated by chromatography and paper electrophoresis. The absence of N-acetylglucosamine, together with the fact that the cell wall is completely soluble in hot alkali, eliminates the possibility of beta-

Table 35

Effect of lysozyme on actinomycete cells (Sohler, Romano and Nickerson)

Organisms lysed by lysozyme	Strain No.	Organisms not lysed by lysozyme′	Strain No.
Streptomyces		*Mycobacterium*	
S. alboflavus	3008	*M. phlei*	23
S. albus	3448	*M. smegmatis*	607
S. antibioticus	3435	*Nocardia*	
S. aureus	3484	*N. asteroides*	3599
S. bobiliae	3310	*N. convolutus*	3414
S. californicus	3312	*N. corallina*	3408
S. chrysomallus	3657	*N. farcinica*	3301
S. coelicolor	3442	*N. paraffinae*	3410
S. craterifer	3373	*N. polychromogenes*	3409
S. erythreus	3036	*N. rubra*	3639
S. flavus	3321	*Streptomyces*	
S. fradiae	3535	*S. aureofaciens*	3550A
S. fulvissimus	3665	*S. lavendulae*	3516
S. globosus	3736	*S. lavendulae*	3530
S. griseus	3475	*S. lavendulae*	3440-8
S. griseus	3492	*S. lavendulae*	3440-14
S. ipomoea	3476	*S. roseochromogenes*	3816
S. lipmanii	3331	*S. venezuelae*	3534
S. olivaceus	3335	*S. viridochromogenes*	3356
S. parvus	3686		
S. praecox	3374		
S. purpurescens	3660		
S. reticuli	3344		
S. rimosus	3558		
S. rimosus	3560 variant		
S. scabies	3649		
S. violaceus	3497		

linked chitin being a constituent of the cell wall. Free hexosamine was not released by lysozyme to any appreciable degree by the action of the enzyme. This was in contrast to the action of lysozyme on gram-positive bacteria, whereby N-acetylglucosamine is released. Before hydrolysis, all the hexosamine in the solution resulting from lysozyme action was found in the undialysable fraction. On the basis of these findings, it was concluded that glucosamine exists in the cell wall of *S. fradiae* as a polymer, which is not associated with cell wall protein after the action of lysozyme.

It was suggested that these facts might be of taxonomic use in distinguishing nonsporulating strains of *Streptomyces* from *Nocardia* and sporulating strains of *Nocardia* from *Streptomyces*. A study of *N. asteroides* 3573, a sporulating strain which in culture could easily be mistaken for a streptomyces, revealed that the carbohydrate composition of the cell wall was that of a typical nocardia, since both arabinose and galactose were the principal sugars found. Similarly, *S. bobiliae* 3310, a nonsporulating strain of streptomyces that can be mistaken for a nocardia, gave a cell wall composition that was typical of a streptomyces.

Considerable variation was found by Sohler in the carbohydrate content of the cell walls of microorganisms. The carbohydrate content of most actinomycetes was about 20 per cent, on the basis of total reducing sugar.

TABLE 36

*Amino acids found in hydrolyzates of actinomycete cell walls** (Sohler, Romano, and Nickerson)

Amino acid	*S. fradiae* (3535)	*S. griseus* (3492)	*N. rubra* (3639)	*Micromonospora* (3452)	*N. polychromogenes* (3409)
Alanine	++++	++++	++++	++++	++++
Arginine	+	+	+	++	++
Aspartic acid	++	++	++	++	++
Cysteic acid	0	0	0	+	0
Diaminopimelic acid	++++	++++	++++	++++	++++
Glycine	+	+	+	+	+
Glutamic acid	+++	++++	+++	++++	+++
Lysine	+	+	+	++	++
Phenylalanine, leucine, isoleucine	+++	++++	++	++	+++
Proline	0	0	0	0	±
Serine	+	+	+	+	+
Threonine	++	++	++	++	+
Tyrosine	+	0	0	+	+
Valine	++	+++	++	++	+
Hexosamine	+++	++	+	++	+

* ++++ = indicates large, intensely colored spot on chromatogram; +++ = indicates a fairly intense spot; ++ = a small definite spot; + = a faint spot; ± = doubtful; 0 = no spot was found, amino acid absent.

In *Micromonospora* the carbohydrate content was somewhat lower, about 16 per cent. On the basis of the percentages of various carbohydrate components present, the cell walls of actinomycetes were found to resemble most closely those of corynebacteria.

TABLE 37

Carbohydrate composition of actinomycete cell walls (Sohler)

Organism	Total reducing sugar*, %	Pentose, %	Hexosamine, %
S. fradiae 3535	22.0	0	19.8
S. griseus 3492	21.6	0	19.9
S. bobiliae 3310	18.8	0	12.0
S. lavendulae 3416	13.2	1.5	3.0
S. roseochromogenes 3816	27.2	1.2	14.8
N. rubra 3639	18.1	10.5	2.6
N. asteroides 3573	17.6	12.2	2.6
N. polychromogenes 3409	23.6	10.3	3.1
Micromonospora sp. 3452	15.9	1.8	14.8

* Determined as glucose.

The carbohydrate composition of the cell walls of *N. rubra*, *N. polychromogenes*, and *N. asteroides* was found to be markedly similar. The major carbohydrate component was a pentose, which comprised about 10 to 12 per cent of the cell wall. A small amount of hexosamine was found, representing 2.5 to 3 per cent of the cell wall. Varying amounts of hexose were present. The total reducing sugar values vary from 18 to 20 per cent. Both the pentose and the hexose have been tentatively identified in all three organisms by paper chromatography as galactose and arabinose. The possibility that the pentose might be due to nucleic acid, indicating the presence of intracellular contamination of the cell wall preparation, was considered. However, an absorption spectrum on the hydrolysate failed to show a peak at about 260 mμ, which is characteristic of purines and pyrimidines present in nucleic acid. The pentose was therefore not due to intracellular contamination (Tables 38 and 39).

Hoare and Work found among the chemical constituents of actinomycetes the LL isomer of diaminopimelic acid (DAP) in the hydrolyzates of whole streptomyces cells, with small amounts of the *meso* isomer. The latter occurred in nocardia cells and in myco-

TABLE 38

Sugars identified in the cell walls of actinomycetes (Sohler)

Organism	Sugars present
S. fradiae	Glucosamine, hexose
S. griseus	Hexosamine, hexose
S. bobiliae	Hexosamine, hexose
S. lavendulae	Uronic acid
S. roseochromogenes	Mannose, galactose, hexosamine
N. rubra	Arabinose, galactose
N. polychromogenes	Arabinose, galactose
N. asteroides	Arabinose, galactose
Micromonospora sp.	Hexosamine, hexose

TABLE 39

Nitrogen, sulfur, and phosphorus content of actinomycete cell walls (Sohler, Romano, and Nickerson)

Concentration, per cent	*S. fradiae*	*S. griseus*	*N. polychromogenes*	*Micromonospora* sp.
Total N	6.7	7.1	8.4	8.1
Hexosamine N	1.5	1.6	0.2	1.2
Protein N*	5.2	5.5	8.2	6.9
Protein (6.25 × protein N)	32.5	34.4	51.2	43.1
Total S	0.2	0.2	0.3	0.4
Sulfur: protein ratio	0.6	0.6	0.6	0.9
Total P	1.3	2.7	0.5	1.3

* Protein N = total N − hexosamine N.

bacteria, with traces of LL isomer in some species. In micromonospora, both isomers were present in similar proportions, with possibly a small amount of DD-diaminopimelic acid.

Diaminopimelic acid was found in a large number of bacteria by Work and Dewey, Cummins and Harris, and Salton. It has not been found in fungi, yeasts, or plants other than the blue-green algae. Cummins and Harris found that glutamic acid, alanine, and diaminopimelic acid are characteristic of most species of corynebacteria. The cell walls of actinomycetes, particularly those of nocardias, resemble the cell walls of corynebacteria.

Work (1957) summarized our recent knowledge of the cell walls of bacteria in general and of actinomycetes in particular. These walls often constitute 25 per cent or more of the cell weight; they are tough and insoluble in all known solvents. Such wall preparations contained lipids, phosphorus, hexosamines, amino acids and usually carbohydrates, but had no nucleic acids, purines, or pyrimidines.

Glucose, mannose, and galactose were found frequently, rhamnose and arabinose more rarely. Glucosamine was always present, while galactosamine occurred in certain species. A hitherto unknown hexosamine was also always present; it was identical with an acidic hexosamine, first found in a product from bacterial spores, provisionally characterized as 3-O-α-carboxyethyl hexosamine and named "muramic acid." The walls probably represent the major, but not necessarily the only, site of diaminopimelic acid; small amounts also occur in soluble cellular contents.

Qualitative examinations of the cell wall constituents were made by Cummins and Harris using a large number of gram-positive Eubacteriales and Actinomycetales. The resulted are summarized in Table 39. They show glucosamine and muramic acid in walls from all organisms, galactosamine in some. Only three or four amino acids were present in each wall preparation; these were glutamic acid, alanine, and either diaminopimelic acid or lysine, and sometimes also aspartic acid, glycine, or serine. Other amino acids, if present, were only in trace amounts. The distribution of the sugars (glucose, galactose, mannose, arabinose, and rhamnose) varied greatly between the species. The conclusion was reached that "each bacterial genus may have a distinctive pattern of cell wall components, in particular among the amino acids present."

TABLE 40

Composition of cell walls of some gram-positive Eubacteriales and Actinomycetales (Work)

Organisms	Amino sugars		Amino acids*			Sugars
	Glucosamine and muramic acid†	Galactosamine	Glutamic acid and alanine	DAP (meso- or LL-isomer) or lysine	Others	
Staphylococcus aureus	+	−	+	Lysine	Gly, Ser	—
Micrococcus lysodeikticus	+	−	+	Lysine	Gly	Gluc
Lactobacillus	+	−	+	Lysine	Asp	Gluc (Gal, Man)
Bacillus	+	−	+	DAP-meso	—	—
Corynebacterium	+	+	+	DAP-meso	—	Gal, Arab, Man
Mycobacterium	+	−	+	DAP-meso	—	Gal, Arab
Nocardia	+	−	+	DAP-meso	—	Gal, Arab
Streptomyces	+	−	+	DAP-LL	Gly	—
Actinomyces	+	−	+	DAP-LL	Gly	—

* DAP = diaminopimelic acid; Gly = glycine; Asp = aspartic acid; Ser = serine; Gluc = glucose; Gal = galactose; Man = mannose; Arab = arabinose.

† Muramic acid = 3-O-α-carboxyethyl hexosamine.

The presence of nucleic acids and phosphorus in the mycelium of actinomycetes has been studied by Guberniev *et al.* The ability of actinomycetes to synthesize polylaevans was studied by Ørskov and Veibel (1938).

Amino Acid Synthesis

Various actinomycetes have been found capable of liberating certain amino acids in synthetic media in which they have grown. Among these acids glutamic acid occupies a prominent place. The maximum concentration of this acid was found by Perlman and O'Brien to range from 0.25 to 1.75 gm/l, corresponding to a conversion of 4 to 29 per cent of the nitrogen in the medium. The presence of glutamic and other amino acids in the extracellular fluids of different species of *Streptomyces* was believed to be due to the autolysis of the cells of the organisms (Gilmour *et al.*

Antibiotic Biosynthesis

Antibiotic biosynthesis has contributed much to our knowledge of the chemical structure of the actinomycete cell. A substantial quantity of streptomycin has been found to occur bound to the cells of the organisms, suggesting that the antibiotic may be a part of the cell wall of the organisms producing it. The bound streptomycin may be released by treatment of the mycelium with acids, with alkalies, or with ionizable salts, but not by the disintegration of the cell by sonic energy, bacteriophage, or enzymatic treatment, as pointed out in Chapter 11. This is true also of the antibiotics streptothricin, neomycin, chloramphenicol, and the tetracyclines. This binding of the antibiotic to the cell does not appear to be a simple ion-exchange phenomenon, since streptomycin added to the mycelium of *S. griseus* was not adsorbed; the binding power of the mycelium is apparently not a function of its weight. Some of the antibiotics, notably the basic compounds, may be considered as polysaccharides, perhaps related to the cell-wall polysaccharides of microorganisms. Other antibiotics, such as chloramphenicol and the actinomycins, may be considered as polypeptides.

These facts do not explain the mode of action of antibiotics upon bacteria and other microorganisms. The sensitivity of actinomycetes to antibiotics produced by other organisms has been used as a method of selecting and identifying such cultures. A certain culture may be sensitive to its own antibiotic to only a limited degree, but it may be much more sensitive to other antibiotics. Some strains of *S. griseus*, however, have been found to be much less sensitive to streptomycin than are many other strains. Exposure to streptomycin has been used as a method of selection of new cultures, presumably higher-yielding types.

An examination of data on the efficiency of conversion of substrate to streptomycin indicates that approximately 15 per cent of the carbon added to the medium, either as carbohydrate or as glycerol, may be converted to streptomycin. The actinomycetes producing chlortetracycline and chloramphenicol are also capable of incorporating into the respective antibiotics substantial quantities of the chloride ion present in the medium. It is also probable that the grisein-producing strains of *S. griseus* do likewise with iron. Gottlieb and Anderson (1947) have shown that the peak of streptomycin production lagged behind the growth peak. The presence of oxygen was essential for streptomycin synthesis (See also Woodruff and Ruger, Eiser and McFarlane, Christenson *et al.*).

The effect of nutrition of *S. griseus* upon streptomycin production has been studied by Bennett (1947). The effect of different amino acids on the biosynthesis of streptomycin is illustrated in Table 41.

The biosynthesis of chlortetracycline by *S. aureofaciens* has been studied by DiMarco (1956), and of erythromycin by Corum *et al.* (Table 42). The effect of specific nutrients upon the formation of different actinomycins and upon the constituent forms of the same actinomycins has been examined by Brockmann and his group, by Schmidt-Kastner, and by Katz *et al.* (1958).

Hunter *et al.* have shown, by the use of $C^{14}O_2$, that the carbon of the guanidine side chains in streptomycin is derived largely, if not entirely, from CO_2. Further information on the mechanism of biosynthesis of antibiotics has been given by Gwatkin, Herold *et al.*, Petty and Matrishin, Yagashita and

TABLE 41

Effect of different amino acids on growth and streptomycin production by S. griseus (Spilsbury)

Nitrogen source	Growth at 7 days	Streptomycin at 7 days, μg/ml
Ammonium nitrate	Good coverage	100
Glutamine	Mycelium white with a mealy appearance	38
Histidine	Medium to good coverage, but mycelium tends to become brownish	20–30
Glycine	Medium to good coverage, but mycelium tends to become brownish	20–30
Alanine	Medium to good coverage, but mycelium tends to become brownish	60–70
Proline	Coverage decidedly poor, mycelium scanty and brownish	18–20
Leucine	Coverage decidedly poor, mycelium scanty and brownish	18–20
Valine	Coverage decidedly poor, mycelium scanty and brownish	30
Isoleucine	Coverage decidedly poor, mycelium scanty and brownish	30
Tyrosine	Coverage decidedly poor, mycelium scanty and brownish	16
Arginine	Very poor growth, mostly submerged	18–20
Phenylalanine	Very poor growth, mostly submerged	16
Methionine	Toxic; growth nil	0

TABLE 42

Chemical changes in the synthetic medium inoculated with S. erythreus (Corum *et al.*)

Days	Erythromycin, μg/ml	pH	Mycelial volume*	Nitrogen, mg/ml			Total carbohydrate, mg/ml
				Total	Amino	Ammonia	
0	0	7.1	0	2.70	2.90	0.07	77.0
2	6	6.4	1.1	2.50	2.70	0.09	72.5
3	65	6.3	4.9	1.75	1.95	0.10	48.0
4	154	5.9	5.8	1.45	1.45	0.05	32.0
5	294	7.1	5.8	1.0	0.90	0.13	16.9
6	412	8.3	6.5	0.7	0.55	0.46	2.4
7	383	8.7	5.5	1.3	0.75	0.51	1.8

* ml/15 ml of broth.

Umezawa, Makarevitch, and numerous others.

Some very interesting observations on biosynthesis of streptomycin were recently reported by Hockenhull (1958). This antibiotic is regarded as a modified trisaccharide, the components being a substituted cyclohexitol, a previously unknown branch chain sugar, streptose, and L-N-methyl glucosamine. The carbon of these moieties was found by radioactive tracer studies to arise from glucose, the principal carbohydrate source used in the medium. The L-N-methyl glucosamine could be directly assimilated into the molecule. The streptidine arose from carbon dioxide, arginine being considered as an intermediate. The L-glucosamine moiety was believed to arise as an inversion of the glucose molecule by way of a cyclohexitol.

Growth of the streptomycin-producing organism takes place under conditions of ample carbohydrate and restricted nitrogen supply, usually in the form of soya-bean meal. A low inorganic phosphate level and high aeration bring about a "Pasteur effect," restricting the formation of organic acids or metabolism by way of the tricarboxylic acid cycle mechanism. This results in an accumulation of phosphorylated carbohydrate intermediates and a locking up of inorganic phosphate, which can then be regenerated by polymerization. Increase of phosphate causes sugar usage to increase and the streptomycin yield to fall. Restriction of aeration brings about the formation of lactic acid.

Doskočil (1958) attempted to elucidate biosynthesis of oxytetracycline by *S. rimosus* in a medium consisting of glucose, starch, corn-steep, $(NH_4)_2SO_4$, $CaCO_3$, and sodium chloride. Two distinct growth phases were observed. In the first phase, glucose was used up and maltose accumulated. Glucose disappeared in 9 to 12 hours of cultivation. This coincided with maximum respiration and maximum level of pyruvate. Interruption of the growth phase is accompanied by fragmentation of mycelium and the beginning of oxytetracycline production. During growth the washed mycelium metabolized only glucose; within six hours after the depletion of glucose it acquired the ability to utilize maltose. The mycelium began to grow again, with no parallel increase of respiration and pyruvate, but with rapid production of oxytetracycline (1.8 to 2 mg/ml).

On a medium with glucose and without starch there was a continuous increase of respiration and formation of pyruvate until complete depletion of glucose; production of oxytetracycline reached only 0.7 to 0.9 mg/ml. The washed mycelium utilized only glucose, not maltose. The adaptation to maltose requires a period of several hours during which the mycelium does not grow and the filaments undergo fragmentation. Presence of glucose inhibits adaptation to maltose. The nature of adaptive enzyme seemed to be different from maltase. For a maximum production of the mycelium, a switch to a slower and more economical metabolism of carbohydrate, such as that of maltose in the second growth phase, is essential.

Antigenic Properties of Actinomycetes

Considerable attention has been centered, in recent years, upon the antigenic properties of actinomycetes. Biagi (1904), in looking for a suitable system for classifying actinomycetes, other than their morphology and physiology, decided to study their serological behavior. He immunized rabbits with five aerobic forms and made cross-agglutination studies. Homologous and heterologous agglutination of low titer was obtained, but the tubercle bacillus was not agglutinated by any of the antisera. Calendoli (1905) was also able to show some cross-agglutination reactions between two actinomycete cultures. Choukevitch (1909), working with organisms that appeared to belong largely to the genus *Nocardia*, obtained an antiserum, by intravenous inoculations, that would agglutinate various strains. Leao (1928) used sera from human actinomycosis cases and an antigen from one of the isolated cultures; positive agglutination was obtained in a titer of 1:160, complement fixation being positive, and precipitin tests negative. Holm (1930) divided a group of strains of the anaerobic *Actinomyces* into two subgroups on the basis of cross-agglutination.

Aoki (1936) has shown that complement-binding reactions of actinomycetes corresponded fully with their agglutination properties. Nine agglutinating types were established, one of which was anaerobic and the others aerobic. Two of the aerobes appeared to be of the nocardia type and six of the streptomyces type. The anaerobic and the first two aerobic forms gave clear agglutination reactions; for the other six, agglutination could be demonstrated only with difficulty. The spores of the latter appeared to contain more of the agglutination receptors than did the mycelium.

Breley (1933) obtained such highly erratic results for complement fixation and precipitation that he concluded, "Nocardia and Streptothrix are bad antigens *in vivo* and *in vitro*." Lieske's results also tended to question the significance of the results obtained by this method in differentiating various groups of actinomycetes. Lentze (1938), as well, drew attention to the technical difficulties involved in carrying out agglutination experiments with actinomycetes, because of frequent spontaneous agglutination. He demonstrated the existence of R and S strains among the cultures isolated from clinical cases of actinomycosis. Goyal (1937) studied a number of cultures of actinomycetes, representing at least three of the genera now recognized; all of them were designated, however, as strains of *Streptothrix*. He came to the conclusion that an extract, comparable to tuberculin and designated as streptothricin, had the same antigenic properties as similar extracts of the tuberculosis and diphtheria organisms. He suggested the presence of antigens common to all these groups. This did not agree, however, with the results of other investigators. Claypole (1913), for example, demonstrated that sera from aerobic pathogenic nocardia and human tubercle bacillus would show, by complement fixation tests, a certain degree of quantitative differentiation between the antigenic substances of these organisms.

Erikson (1940) established that the anaerobic human (*A. israeli*) and bovine (*A. bovis*) strains were serologically related within themselves, but there was no cross-reaction with representative aerobic actinomycetes, saprophytes or parasites. Ludwig and Hutchinson (1949) reported that serological procedures can be used as an aid in the identification of actinomycetes. Slack *et al.* made a comprehensive study of the agglutination reactions among the various genera of the actinomycetes. They demonstrated that the microaerophilic forms are serologically related. The presence of common group antigens was indicated. Slack *et al.* (1955) divided the microaerophilic forms isolated from different sources into two serological groups, thus establishing that habitat does not correlate with antigenic composition.

Solovieva *et al.*, Yokoyama and Hata, and Okami are among others who studied the serological reactions of actinomycetes. Gonzalez-Ochoa and Vasquez-Hoyos (1953) were able to divide the pathogenic actinomycetes into four groups on the basis of their serologic relations: (a) the *bovis* group, including also certain *Nocardia* species; (b) the *somaliensis* group; (c) the *madurae* group; and (d) the *paraguayensis* group, the last showing serologic relations with the streptomyces isolated from soil, namely *S. albus*, *S. griseus*, and *S. lavendulae*.

The fact was always emphasized that many of the difficulties involved in the analysis and interpretation of the results of the antigenic and serological reactions of actinomycetes are due to lack of certainty as to the organisms used by an investigator. Okami (1956) was able to demonstrate that agglutination techniques offer promising tools in the classification of *Streptomyces* species.

Wodehouse and Backus suggested an ingenious method for utilizing the antigenic properties of actinomycetes for taxonomic purposes. The double diffusion technique on agar media was used. Precipitation bands are formed when the diffusion from antigenic sources encroach upon that of antiserum. If two extracts are alike, their bands join to form arches above the serum source. If they are unlike, they cross each other without interference. Various degrees of relationship between organisms can thus be obtained. There was but little overlapping with the other genera of the *Actinomycetales*.

Other Biochemical Activities

Because of their special significance, several other biochemical properties of actinomycetes are treated in detail in separate chapters. These include the formation by actinomycetes of enzymes, of various lytic mechanisms, pigments, vitamins, and antibiotics. The antibiotics are receiving particular consideration because of their growing practical importance (Chapter 15, and Chapters 31–36, Volume III).

CHAPTER 10

Lytic Mechanisms

Lysis of microbial cells in general and of actinomycete cells in particular comprises a number of reactions, some of which frequently are considered as enzymatic processes. These can be classified into three groups: (a) autolytic reactions, in which the growing cells, at a certain stage of their development, undergo lysis; (b) bacteriolytic processes, whereby certain complex mechanisms involving also enzymatic reactions produced by actinomycetes have the capacity to dissolve the cells of various bacteria, living or dead; (c) sensitivity of actinomycetes to phages, designated as actinophages and specific for each organism. The first two groups of lytic reactions, or those of autolysis and bacteriolysis, are known to occur not only among bacteria and actinomycetes, but also in yeasts and filamentous fungi. The sensitivity to phages, however, is characteristic, so far as we now know, of only bacteria and actinomycetes.

Actinomycetes also possess certain other lytic mechanisms. It is sufficient to mention, for example, the ability of various forms to cause the lysis of red blood cells. The antibiotics produced by actinomycetes may bring about reactions involving lysis of various microorganisms; these mechanisms fall into a group by themselves and are treated in detail in Chapter 15.

Autolysis

One of the most characteristic properties of various actinomycetes is their ability to undergo lysis. The animal pathogens were the first to receive consideration for their capacity to lyse at certain stages of growth. Later, this phenomenon was shown to hold true also for plant pathogens. Finally, some of the soil saprophytes were found to possess the same property.

The mechanisms responsible for the processes of autolysis among actinomycetes have been variously called lysins, autolysins, actinolysins. Dimitriev first described, in 1934, the phenomenon of lysis among pathogenic actinomycetes isolated from actinomycosis of man. The capacity for lysis of actinomycete colonies grown on nutrient media was studied by Dimitriev and Firinkov, who came to the conclusion that this capacity is proof of the bacterial nature of actinomycetes.

Dimitriev and Souteeff (1936) later reported that when an actinomycete culture, grown on agar media, underwent lysis, the phenomenon was associated with the formation of a certain type of colony. Two types of daughter colonies were produced as a result of lysis: one was similar to the mother colony and characterized by its capacity for lysis; the other did not lyse and was morphologically different from the first. The cultures originating from the colonies capable of undergoing lysis were strongly proteolytic and produced no aerial mycelium. The nonlysing colonies yielded cultures that were less proteolytic; they formed a chalky white aerial mycelium; the reaction of the medium was changed to alkaline. In broth cultures, lysis took place in 2 to 3 weeks, and was associated with the formation of a nonenzymatic and nontransmissible lytic factor.

Dimitriev and Soutiev (1947) suggested that the lysed preparation of pathogenic actinomycetes, designated as actinolysate, could be utilized in the therapy of infections caused by these organisms. They emphasized, however, that such preparations must first be purified and concentrated.

Krassilnikov and Koreniako (1938) studied the phenomenon of autolysis among those actinomycetes that are now known to belong to species of *Streptomyces* and *Nocardia.* In the streptomyces, either the entire colony underwent lysis or only partial lysis occurred, usually beginning in the center of the colony and proceeding to the periphery; the colony became slimy, flat, and transparent, finally changing to a viscous consistency. In the nocardias, the colonies were lysed simultaneously throughout the entire surface. The phenomenon of autolysis was not accompanied by the formation of saltants or new races. The lytic substances were produced by the cells themselves and were not of outside origin. Autolysis usually began at the time of aging of the culture; at 60 to 70°C, complete lysis occurred in a few minutes. Any factors retarding growth of the organism were found to hasten lysis. Thus, a culture of *N. citrea* underwent lysis under suboptimal conditions of temperature, or under the influence of ether, chloroform, benzol, acetone, and other volatile compounds. Autolysis was also found to be hastened by the secretion products of certain spore-forming bacteria. The rapidity of lysis caused by these factors varied from a few minutes (at 60°C) to several hours.

The agent responsible for autolysis was resistant to heat; even 1 hour at 80°C had no effect but it was inactivated in 5 minutes at 100°C. In contradistinction to phage, it acted not only upon living cells but also upon dead cells. The conclusion was reached that the autolytic substance of actinomycetes is produced in the cells themselves and is liberated at the moment of their decomposition.

Krassilnikov and Koreniako suggested a resemblance of autolysis among actinomycetes to phage formation by bacteria. The lytic factor of actinomycetes was found to be highly specific, since it had no effect upon other species or even upon other strains of the same organism; it was thus distinguished from lysozyme. Different strains of an organism underwent lysis with varying degrees of rapidity. Production of the lytic factor or its mode of action was believed to be different for the different organisms.

Of the 1000 or more freshly isolated cultures studied by Krassilnikov, only a few were able to undergo autolysis. A culture of an organism that gave, when freshly isolated, heavy compact growth covered with white aerial mycelium, produced, on continuous transfer, flat, smooth, and somewhat moist colonies that lost the property of forming aerial mycelium. Gradually the growth of the culture was reduced to a thin slimy film, and, on repeated transfer, became transparent, until the culture finally ceased to grow altogether; all attempts to keep it alive were unsuccessful. Other cultures of actinomycetes belonging to different morphological and physiological groups showed similar lysis, although in different degrees.

A colony may not undergo complete lysis. Only certain sectors or spots may dissolve, the unlysed portion of the colony remaining unaffected. Frequently, lysis begins in the center of the growth and proceeds to the periphery. The mechanism of lysis among the pathogenic actinomycetes is similar to that occurring among the saprophytic forms, but the rate of lysis is more rapid. Organic media are favorable to the lytic process. When a culture having the capacity to undergo lysis is grown on plates at 25°C, then incubated

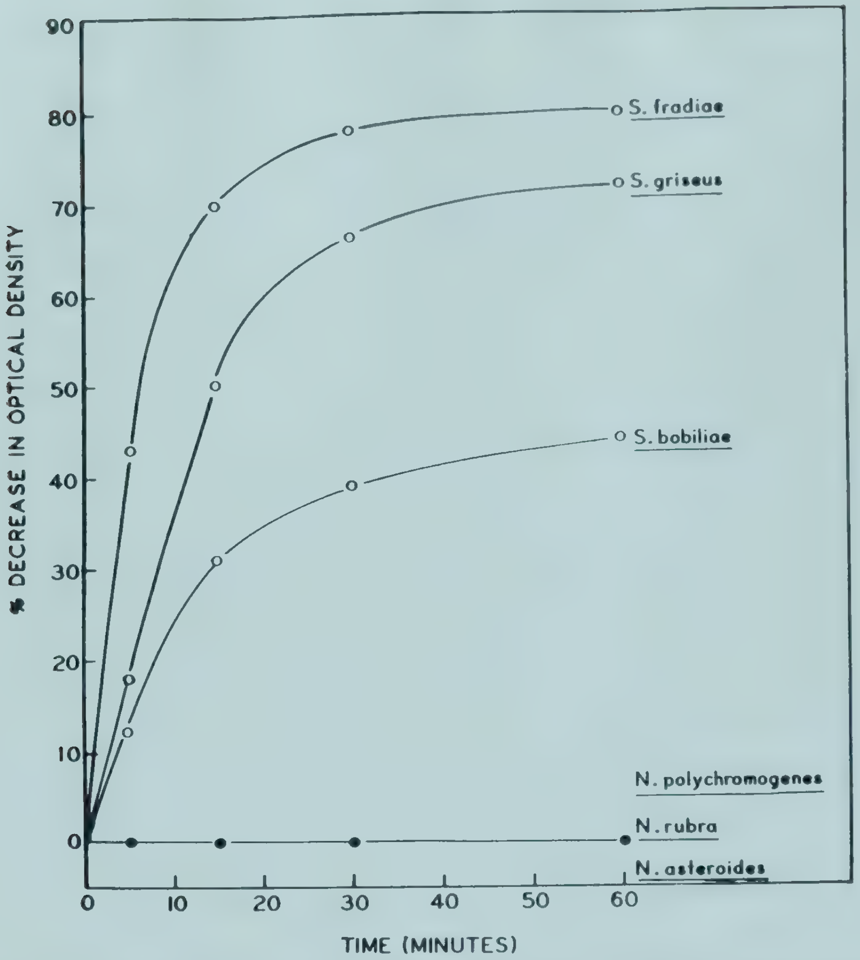


FIGURE 73. Lysis of cell walls of *Streptomyces* vs. *Nocardia* by lysozyme (Reproduced from: Romano A. H. and Sohler, A. J. Bacteriol. 72: 866, 1956).

at 30 or 37°C, lysis takes place in 4 to 6 hours. Not all the mycelium is lysed uniformly, some of the hyphae producing chlamydospores, spherical bodies, or other fragmentary material. Under favorable conditions, these bodies are able to grow and develop into fresh colonies.

Katznelson isolated from manure composts a culture of a thermophilic actinomycete, which grew well on organic media. When transferred to a synthetic medium containing ammonium sulfate and starch, it underwent lysis after 24 to 48 hours of incubation at 50°C. During growth, the culture became acid. Addition of $CaCO_3$ to the culture prevented lysis. Stanier reported that an agar-decomposing strain of *S. coelicolor* underwent rapid autolysis and soon died out. Lysis took place when the organism entered the sporulating stage.

The process of autolysis in cultures of *S. griseus*, the streptomycin-producing organism, has been studied extensively. When the growth of this organism in submerged culture reaches a maximum, lysis sets in and fragmentation of the mycelium occurs. The peak of streptomycin production lags somewhat behind the growth peak. The changes in reaction of medium take place in two phases. One occurs during growth, when the medium becomes acid, and one is associated with lysis of the culture, when the medium becomes alkaline and may reach a pH of 8.6.

Dulaney *et al.* (1947) differentiated two phases of metabolic activity of *S. griseus*, grown under shaken conditions:

1. The growth phase, characterized by formation of mycelium, reduction of soluble nutrients in the medium, production and utilization of lactic acid, a high oxygen demand, and little production of streptomycin.
2. The autolytic phase, characterized by a marked decrease in weight of mycelium, an increase in inorganic phosphorus and soluble nitrogen in the medium, a drop in oxygen demand to zero, and production of large quantities of streptomycin.

Increases in carbohydrate or phosphate content of the medium have but little effect on the general changes, although they may lengthen the growth phase. Various strains show no significant differences in the metabolic changes, although they may show marked differences in streptomycin production.

Schatz and Waksman (1945), studying the production of streptomycin by different strains of *S. griseus*, observed that colonies devoid of aerial mycelium formed no streptomycin (Dulaney found exceptions). Such colonies gave rise to cultures that underwent much more rapid lysis than the normal cultures with aerial mycelium. In the practical production of streptomycin, it is generally observed that, under submerged conditions of growth, maximum formation or accumulation of the antibiotic corresponds to the beginning of lysis; advanced lysis usually results in a rapid destruction or inactivation of the streptomycin already produced.

In further studies, it was found that *S. griseus* may give rise to two types of inactive strains. One of these is free from aerial mycelium. In culture, especially in a submerged condition, it undergoes rapid lysis. It gives rise to an acid reaction in the medium and yields a viscous broth. This strain is sensitive to the antibiotic action of streptomycin and is comparable in that respect to other inactive actinomycetes, whereas the streptomycin-producing strains are highly resistant to the action of this antibiotic. This variant is similar to the active culture in such cultural characteristics as lack of dark pigmentation on organic media, proteolytic action, and hemolytic capacity. By proper culture and selection, this asporogenous strain can be made to revert to the sporulating strain, which will have the capacity of producing streptomycin. Some asporogenous strains are definite mutants, according to Appleby.

According to Lumb, *S. griseus* undergoes lysis more rapidly under submerged conditions of growth than under stationary conditions. The culture tends to become viscous as a result of formation of the lysed material. *S. fradiae*, the culture that produces neomycin, behaves in the same way. Lumb also observed that the accumulation of the antibiotics corresponds to the beginning of lysis.

Various attempts have been made to correlate the phenomena of autolysis among the actinomycetes and their bacteriolytic action upon gram-positive bacteria. Although both seem to involve the hydrolysis of proteins, bacteriolysis involves lower proteolytic activity, is active at a different pH level, and proceeds further than autolysis.

Gorjunova considered the process of lysis among actinomycetes as enzymatic in nature, since it is accompanied by the breakdown of proteins. According to her, the lytic agents consist of two components that can be separated by dialysis. Neither of these is active by itself, but when the two are mixed, they bring about the lysis of the cultures.

Bacteriolysis

The first observations on the lytic effect of actinomycetes upon other organisms were made by Gasperini in 1890. He reported that actinomycetes are able to grow on the surface of cultures of bacteria and fungi, living as a sort of parasite upon these organisms, and are capable of digesting them.

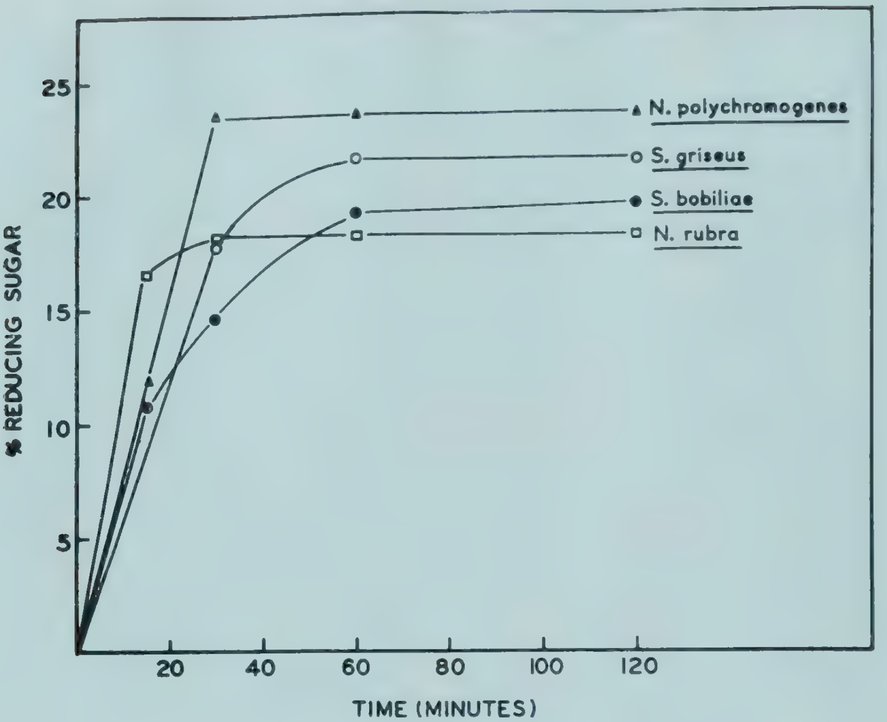


FIGURE 74. Liberation of reducing sugars (expressed as per cent of dry weight of cell walls), on 1 *N* HCl hydrolysis at 100°C of cultures of *Streptomyces* and *Nocardia* (Reproduced from: Romano, A. H. and Sohler, A. J. Bacteriol. **72:** 867, 1956).

Lieske reported in 1921 that certain actinomycetes have the capacity to dissolve bacteria and other microorganisms. A suspension of dead bacteria, such as staphylococci, was mixed with agar and poured into plates. These plates were inoculated with actinomycete cultures and incubated. The actinomycetes were able to dissolve the bacterial cells, as shown by the clear zones produced around the actinomycete colonies. Lieske demonstrated further that living bacteria likewise may be dissolved; both spore-forming and nonspore-forming gram-positive and gram-negative bacteria were sensitive to the action of certain actinomycetes. Lieske considered this effect to be due to bacteriolytic mechanisms of an enzymatic nature. It may be of interest to quote Lieske, because of the present great significance of the lytic mechanisms of actinomycetes. It is of particular interest to note that he questioned, although he did not exclude, the therapeutic potentialities of this phenomenon. He said:

"Ob die bakteriolytischen Enzyme der Strahlenpilze einmal von therapeutischer Bedeutung werden können, ist fraglich, aber immerhin nicht ausgeschlossen. Die grossen Erwartungen, die man auf die Pyocyanase, den entsprechenden Stoff des Bacterium pyocyaneum gesetzt hat, sind leider bisher nicht erfüllt worden. Dass mit den Enzymen der Strahlenpilze, die jedenfalls eine bedeutende bakterizide Wirkung haben, bei Anwendung geeigneter Methoden bessere Ergebnisse erzielt werden können als mit Pyocyanase, ist keineswegs unmöglich."

In 1928, Lieske again compared the phenomenon of bacterial lysis brought about by cultures of actinomycetes with the action of pyocyanase, a bacterial product, not enzymatic in nature. He noted that no further study was made of the actinomycete preparation (which he considered to be an enzyme) and that it was not known whether it, like pyocyanase, could be used for curative purposes.

In 1924, Gratia and associates suspended dead cells of staphylococci and of other bacteria in agar and exposed the plates to the air. A white culture of an actinomycete, designated as "Streptothrix," but definitely a *Streptomyces*, developed on the plates. When this culture was transferred to a suspension of dead staphylococci in sterile saline solution, the bacterial suspension became clarified in 36 hours, accompanied by flaky growth of the actinomycete. When the lysed emulsion was filtered, it was found capable of dissolving a fresh suspension of dead bacteria. This culture could attack all staphylococci tested, as well as certain other bacteria, such as *Ps. aeruginosa*; however, it was inactive upon *M. tuberculosis* and *E. coli*. The lysed material was designated as a *mycolysate*. It did not possess the toxicity of a nonlysed suspension, but retained its antigenic properties. Gratia later asserted that the "Streptothrix" culture was also able to attack living cells of bacteria, except *E. coli* and *Eberthella typhosa*, which had to be first killed by heat.

In 1935, Borodulina demonstrated the ability of certain actinomycetes to dissolve various spore-forming bacteria, especially *Bacillus subtilis*. She emphasized that the lytic substance of actinomycetes is produced in acid media but not in basic. The substance was resistant to heat, although it was destroyed in the autoclave. Nakhimovskaia (1937) also demonstrated the ability of various actinomycetes to form a lytic substance, which is excreted into the medium. The substance inhibited the growth of various bacteria and even dissolved them.

Krassilnikov and Koreniako (1939) considered this property of actinomycetes to lyse certain bacterial cells as due to lysozyme, which was first described by Fleming in 1922. Kriss (1940) isolated from a culture of *S. violaceus*, a thermostable substance which was designated as *bacteriolysin*. This substance was soluble in water and was believed to be different from bacteriophage and identical with lysozyme. Kriss reached the rather significant conclusion that actinomycetes produce various substances that act upon bacteria either in an antagonistic or in a lytic manner.

The most extensive studies on the bacteriolytic activities of certain actinomycetes have been made by Welsch, following in the footsteps of Gratia and his associates. Beginning in 1936, he and his collaborators have published a large number of papers on this lytic principle.

Welsch first obtained a bacteriolytic preparation from a culture of *S. albus*. It possessed strong activites and was heat-labile; it was destroyed at 65° in 2 hours and at 80° in 5 minutes. At low temperatures, it remained stable for many months; at pH 4.0, it was destroyed in 24 hours. The active substance was precipitated by ammonium sulfate, ethyl alcohol, and acetone. The production of this bacteriolytic substance was closely related to the sporulation of the organism; there was no relation, however, between its production and autolysis of culture. This substance was designated as *actinomycetin*.

Welsch further reported that some of the activity of actinomycetin in the filtrate was lost on passage through bacterial filters. Three groups of bacteria were recognized in their relation to actinomycetin:

1. Those bacteria which were lysed by the aqueous extract of the agar cultures of *S. albus*, namely, pneumococci and hemolytic streptococci.

2. Bacteria which were not dissolved by the most active soluble substance, but which were depressed by the mycelium of the actinomycete; these included various sarcinae and *B. megatherium*.

3. Bacteria which were not acted upon either by the mycelium or by the active substance. These comprised the colon typhoid-paratyphoid and the pyocyaneus groups. When these bacteria were killed by heat or were placed under conditions unfavorable to

multiplication, they were dissolved by the lytic substance. For instance, cells of *E. coli* acted upon by radium emanation, which stops their multiplication, became susceptible to the lytic substance.

Actinomycetin was shown to consist of a protein-enzyme system, active particularly upon gram-negative bacteria and upon certain living gram-positive bacteria. The precipitated preparation was found also to possess bactericidal properties. This "bactericidin" was believed to exist in the culture filtrate as a harmless substance, which was activated on precipitation.

Further studies brought out the fact that the lytic principles of actinomycetes are rather complex in nature. They frequently contain as many as four substances that differ in their action and in the organisms acted upon. The lysis of living bacteria by actinomycetin preparations was considered to be a result of two factors: (a) one acting upon the living cell, being a bactericidal factor; and (b) the other acting upon dead cells, being a bacteriolytic factor. The second factor is helped along by the process of autolysis. Later, Welsch (1947) designated the bactericidal substance as *ribonucleinase* and the lytic principle as *actinozyme*, which is the enzymatically active protein. This enzyme is excreted by the organism in the process of sporulation. The presence of carbohydrates favors its production in artificial media.

Muggleton and Webb (1952) also demonstrated that the exocellular bacteriolytic system of streptomyces depends upon an enzyme of the ribonuclease type; a deoxyribonuclease was also present in the culture filtrate. McCarty found that the lytic mechanisms of *S. albus* are mucopolysaccharidases.

These bacteriolytic mechanisms are widely distributed among actinomycetes (Welsch, 1954). As many as 29 per cent of all the cultures examined produced staphylolytic mechanisms, and 48 per cent, streptolytic mechanisms; the same preparation may contain both. The antibiotic-producing organisms form no lytic substances or only very limited amounts.

Welsch and Thuysen (1956) summarized the bacteriolytic properties of *S. albus* strain G. Evidence was presented for the existence of several specific systems: 1. A colilytic agent or actinozyme, responsible for the lysis of heat-killed gram-negative bacteria and involved in the dissolution of at least some heat-killed gram-positive organisms; 2. a streptolytic agent, acting upon heat-killed or living streptococci; 3. two pneumolytic agents, one acting upon either heat-killed or living pneumococci, and the other upon the living only; 4. a complex staphylolytic system, dissolving living staphylococci and other bacteria and comprising two components which, after isolation and purification, were found to be specific peptidases acting in synergy upon some unidentified constituent of the cell wall. The enzymes of actinomycetin which are able to dissolve living bacterial cells were considered as true antibiotics.

The mode of action of the staphylolytic systems was likened to that of lysozyme, which also acts upon a constituent of the cell wall of sensitive bacteria. Lysozyme production by actinomycetes has been reported, but differences of specificity between this enzyme and actinomycetin G were pointed out previously. The unsusceptilibity of staphylococci to lysozyme, due to the nature of the cell wall, was considered sufficient evidence for discarding the idea of a lysozyme-like nature of the staphylolytic agent. The nature of the products liberated from cell walls by the staphylolytic system definitely establishes that the peptidases involved are different from lysozyme, which is a polysaccharidase.

As was shown also by Tai and van Heyningen, the colilytic system is a rather specific enzyme, distinct from the proteases of the crude actinomycetin. It possibly acts upon a definite component of the bacterial cytoplasm, access to which is possible only after disruption of the cell wall, as seems to be the case for trypsin.

Swertz made a study of the phenomenon of halo formation in the process of bacteriolysis by actinomycetes. When the actinomycetes were grown on bacterial water agar plates (using heat-killed *E. coli* and heat-killed or living *Staph. aureus* in the agar), the medium was clarified, the zones of bacterial lysis being at times surrounded by a halo of incomplete clarification. This phenomenon was explained by assuming the existence of a staphylococcal factor favorable to lysis by actinomycetes. This factor diffuses from the cells heated to 52 to 56°, or treated with an antiseptic, or dissolved through the action of actinomycetes. Gratia and Dath (1925) also spoke of complete lysis preceded by a preparatory stage in which the microbes are agglutinated and are swollen.

Jones *et al.* have shown that certain actinomycetes possess a system composed of a lipoidal bactericidal substance, a ribonuclease, and a proteolytic enzyme. Killed gram-negative bacteria as well as gram-negative forms of gram-positive organisms are lysed; living organisms are not affected. Gram-positive bacteria are killed only under conditions favorable to autolysis.

The lysis of pathogenic bacteria by streptomyces may be of considerable economic importance. A culture producing a wine-colored soluble pigment and a white to gray aerial mycelium exerted a lysogenic effect upon *Phytomonas*, *Erwinia*, and various other gram-positive and gram-negative bacteria. Potato extract-glucose agar media were particularly favorable to the production of the lytic agent. When the streptomyces culture was added to soil infected with *Phytomonas tabaci*, the plants were protected against infections. This points to the potential importance of such lytic agents in soil processes and their relation to true antibiotics (Darpoux and Faîvre-Amiot).

Pakula and Tye used an actinomycetin preparation for the extraction of deoxyribonucleic acids from bacteria.

Production and Activities of Actinophage

Various actinomycetes can be attacked by filterable viruses or phages, known as actinophages. In this respect, the organisms show a high degree of specificity. These actinophages occur abundantly in nature, particularly in manures and soils (Gilmour and Butala).

Wiebols and Wieringa were the first to observe, in 1936, that cultures of certain actinomycetes, now considered as species of *Streptomyces*, isolated from infected potatoes underwent lysis. This was found to be due to the production of a specific, transmissible phage. Repeated additions of phage-containing culture filtrates to fresh cultures of the organism resulted in the development of a phage which produced a large number of plaques in cultures of the same organism grown on solid or in liquid media. Phages active upon the animal pathogens *A. bovis* and *N. farcinica* were also obtained. One culture produced a polyvalent phage which was also active upon *S. scabies*.

Mülhens (1941) made a study of the mechanism of phage action upon actinomycetes. The whole colony was found to be attacked, beginning at the periphery and proceeding to the center. In some cases the culture had to be repeatedly reinoculated into phage-containing preparations before lysis was attained.

The interest in the phages of actinomycetes received a new stimulus with the discovery of the potentialities of some of these organisms to produce important antibiotics. This first became evident in the production of streptomycin by *S. griseus*.

The sensitivity of streptomycin-producing strains of *S. griseus* to different phages raised

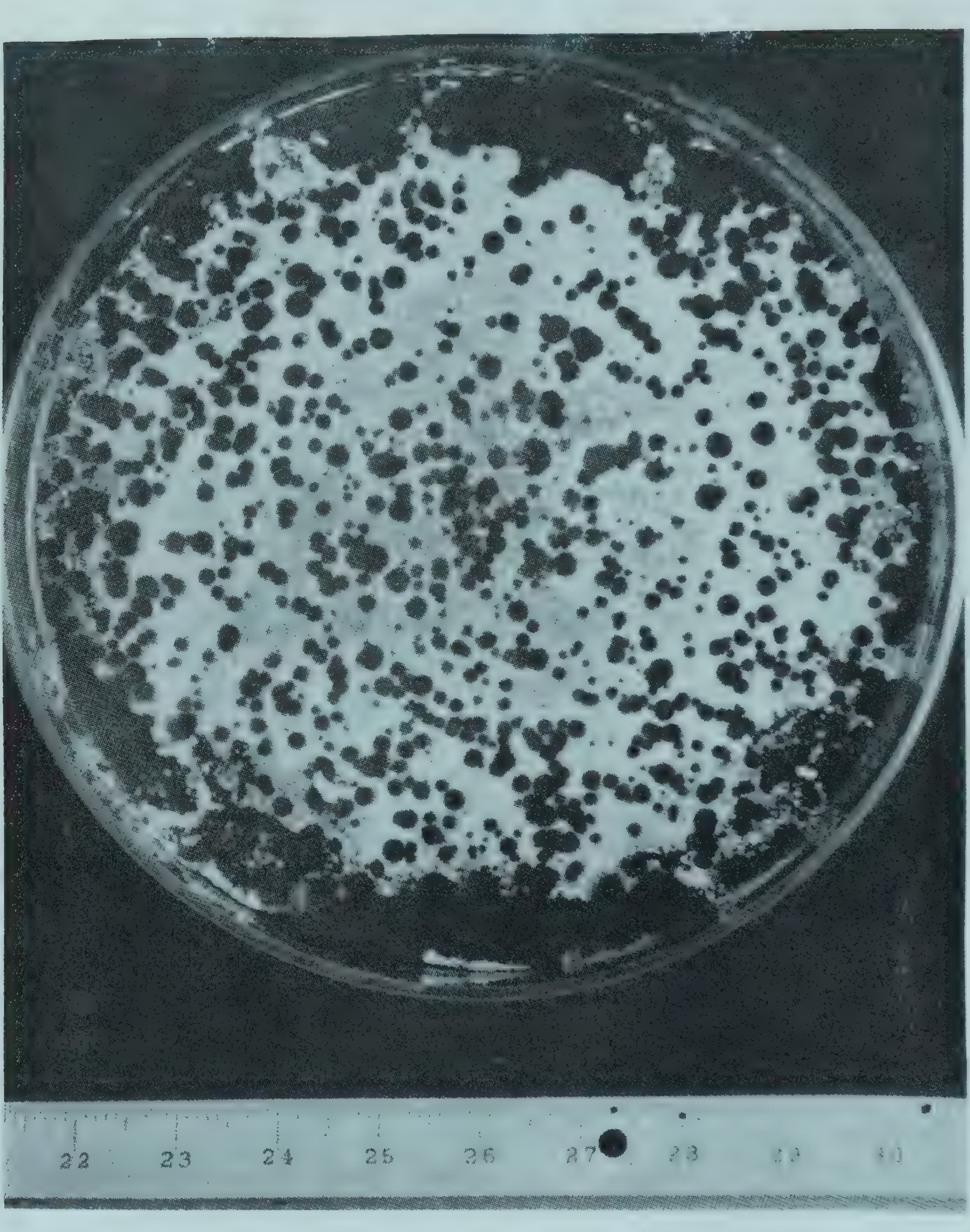


FIGURE 75. Formation of plaques (Reproduced from: Woodruff, H. B. *et al.* J. Bacteriol. 54: 536 1947).

some important economic problems. The lysis of the culture by phage appeared to be quite distinct from that produced by autolytic factors. Saudek and Colingsworth recorded in 1947 that the action of the transmissible lytic agent upon *S. griseus* had all the properties of phage. In young cultures the phage developed rapidly and brought about lysis of the mycelium. The plaque method was used for measuring phage concentration. Streptomycin production was partly or completely stopped by the phage. Cultures resistant to the phage action could easily be isolated from phage-infected cultures.

By exposing submerged cultures of *S. griseus,* in a stationary condition, for 24 hours, Woodruff *et al.* (1947) easily demonstrated the presence of actinophages in the atmosphere. Upon transfer of a filtered broth into a fresh culture of *S. griseus*, phage multiplication was obtained. After six transfers, each phage particle increased to 75×10^{20}. Phage-resistant strains were readily isolated; such cultures retained their capacity to produce streptomycin but were not absolutely free from phage.

To obtain active phages, a 3- to 5-day-old shaken culture of a streptomycin-producing strain of *S. griseus* is filtered aseptically through paper and inoculated on plates. A given phage preparation is inoculated into the young cultures, allowed to incubate for 24 to 72 hours, and filtered through a Seitz filter. Dilutions of phage, ranging from $1:10^6$ to $1:10^{12}$ are added to 10-ml portions of the sterile nutrient agar, previously inoculated with 0.1-ml portions of the paper-filtered culture; the agar is poured into plates; these are incubated at 28°C for 2 days. The plaque counts are then made and calculated for 1 ml of culture. Some preparations gave 4×10^{10} or more particles per milliliter (Koerber *et al.*, Walton).

Reilly *et al.* reported that actinophage attacks only the streptomycin-producing strains of *S. griseus*. No effect was obtained on streptomycin-producing organisms other than *S. griseus*. Strains of *S. griseus* that did not produce streptomycin did not allow phage multiplication; the phage may thus actually be destroyed or adsorbed.

TABLE 43

Effect of addition of phage upon phage multiplication and streptomycin production by different streptomyces in stationary cultures (Reilly, Harris, and Waksman)

S. griseus strain	Phage added*	9 days		13 days	
		Phage per ml $\times 10^7$	Sm, µg/ml	Phage per ml $\times 10^7$	Sm, µg/ml
3463	0	—	—	0	21
	+	—	—	200	5
3475	0	0	30	0	180
	+	>50	<5	370	<5
3480	0	0	31	0	189
	+	10	<5	30	28
3481	0	0	73	0	174
	+	50	<5	260	13
4	0	0	43	0	201
	+	30	<5	160	<5
3475-2PR	0	>0.01	40	40	129
	+	>50	16	370	75
3478	0	0	<5	0	<5
	+	0	<5	0	<5
3326a	0	—	—	0	<5
	+	—	—	>0.2	<5
S. bikiniensis	0	0	<5	0	30
	+	3	30	7	33

* Each 60-ml flask of culture received at start 0.1 ml of M-1 phage, amounting to 7×10^7 particles per milliliter of medium.

The actinophage of *S. griseus* multiples only on living cell material and not on the heat-killed material of this organism. The actinophage has an optimum temperature for multiplication at 28°C. It does not multiply at 37°C or above. Actinophage can withstand a temperature of 75°C for 1 hour, but is completely destroyed at 100°C in 10 minutes. Actinophage can be stored at 6°C without loss of activity, but storage at 28°C or at higher temperatures results in a loss of activity, the rate of loss being proportional to the increase in temperature. The phage is more rapidly destroyed in organic media than in water. It is also inactivated in acid media. It was suggested, therefore, that a phage-infected culture of an actinomycete be grown in an acid medium to free the culture from the phage. The action of phage takes place best in organic media; very little or no action is observed in synthetic media.

Other antibiotic-producing streptomyces are also subject to attack by specific phages. This is true, for example, of the chlortetracycline-producing *S. aureofaciens* (Weindling and Karpos).

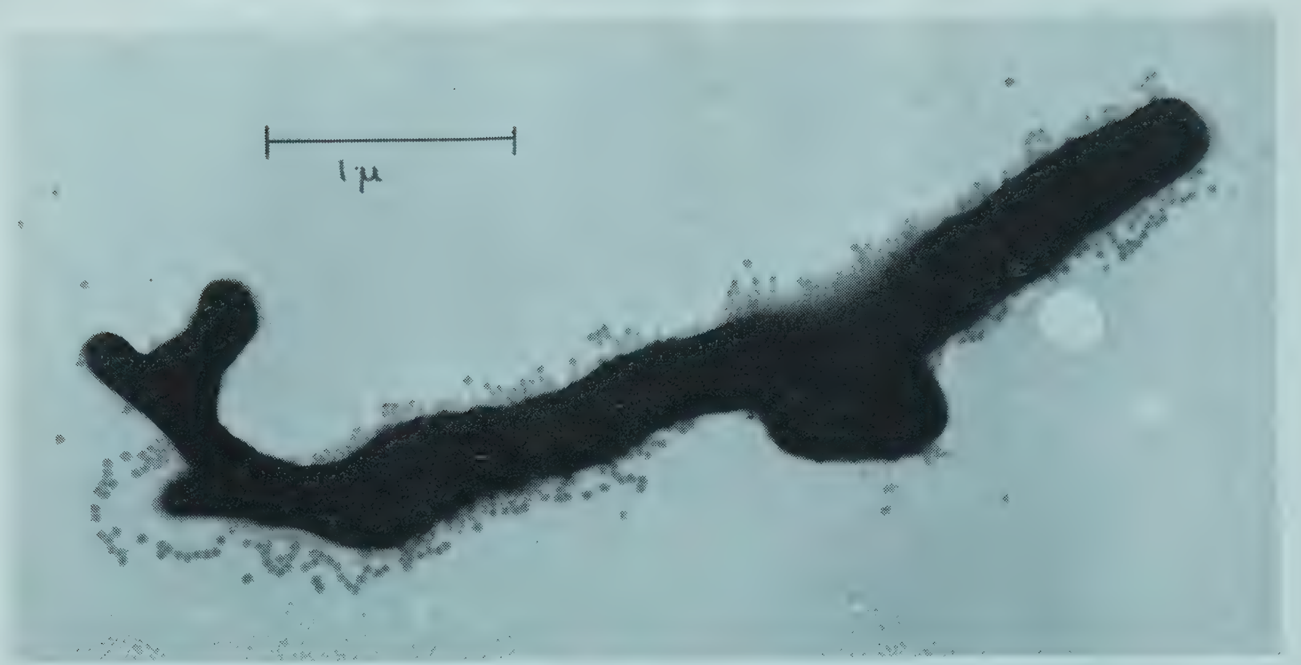


FIGURE 76. Phage particles adsorbed on the surface of a germinating streptomyces spore (Reproduced from: Mach. F. Centr. Bakteriol. Parasitenk. Abt. II, 111: 555, 1958).

TABLE 44

Phage multiplication in cultures of actinomycetes and its effect upon the production of antibiotics (Reilly, Harris, and Waksman)

Organism	Phage* added after hours of incubation	Total incubation, days				
		2	4		6	
		Phage per ml × 10^7	Phage per ml × 10^7	Antibiotic activity, S units/ml	Phage per ml × 10^7	Antibiotic activity, S units/ml
S. griseus 4	Control†	0	0	66	0	90
S. griseus 4	Start	22	650	35	930	48
S. griseus 4	24	9500	7000	96	4600	135
S. griseus 4	48	—	166	120	90	90
S. griseus 3478	Control	0	0	—	—	14
S. griseus 3478	Start	8	1.3	—	—	15
S. bikiniensis	Control	0	0	29	—	18
S. bikiniensis	Start	0.05	0.13	24	0	30
S. lavendulae	Control	—	0	15	—	<10
S. lavendulae	Start	—	8.8	<10	—	<10
N. asteroides	Control	—	—	—	0	—
N. asteroides	Start	—	—	—	9.4	—

* 7 × 10^7 phage particles added per ml of culture.

† No phage was added to control cultures.

Different phages are able to attack the same organism. Cultures of *S. griseus* made resistant to one type of phage may in time become sensitive to another. The formation of lysogenic strains containing a prophage was demonstrated by Welsch (1954). Welsch (1957) further demonstrated that several actinophages can be found in a single natural substrate. They differ in the morphology of their plaques, their host-range, and their antigenic properties. A given actinophage may be present in its natural habitat in a concentration of 10^3 to 10^4 particles/ml.

A search was made for truly lysogenic streptomyces. Thymol-sterilized culture fluids of various organisms were tested upon many indicator strains. About 15 per cent of the organisms freshly isolated from a natural substrate or taken from a collection of cultures actually carried a phage. The true lysogeny of this phenomenon was demonstrated by the constancy of the ratio of phage to streptomyces in different cultures, and by the maintenance of phage production after repeated single colony isolation or serial cultivation in a medium containing a specific antiphage serum.

The theoretical and practical significance of lysogenesis among actinomycetes has been discussed by Rautenstein (1957). The phenomenon of true lysogeny was found to be widely distributed among the actinomycetes. As many as 53 per cent of the *S. olivaceus* cultures examined possessed that property. Numerous other species proved to be lysogenic, including *S. diastaticus*, *S. cacaoi*, *S. candidus*, *S. griseus*, *S. antibioticus*, and *S. scabies*.

Many of the lysogenic actinomycetes easily liberate the phage when grown as submerged cultures for 48 hours on a shaker. In a study of their mutual influence, the lysogenic state of a series of actinomycetes was demonstrated by juxtaposing agar blocks bearing certain cultures on the superficial growth layer of others. Some of the lysogenic cultures contained various phages with different lytic properties.

An indicator culture was used to reveal the development of the phage of the lysogenic culture. It often influenced the lytic properties of a given phage. This influence became particularly significant when the indicator culture was itself lysogenic. Phages which have the faculty to cause lysis of their own culture were often isolated from lysogenic actinomycetes. This suggested that many actinophages are able to change their lytic property more or less easily.

Some phage-resistant variants obtained experimentally differed from their initial cultures in their antibiotic properties. Many of these variants were found to be lysogenic. The possibility was suggested that acquisition of new antibiotic properties may be connected with lysogenization.

Rautenstein suggested that the broad distribution of lysogeny among actinomycetes and the diverse character of changes caused by actinophages in corresponding sensitive cultures influence the variability and the evolution of the actinomycetes in an important manner. The distribution of lysogenic actinomycetes in nature has been studied further by Bradley.

The isolation of actinophage from soil has been studied by Rautenstein and Kofanova (1957), Khavina and Rautenstein (1958), and others. *S. olivaceus* actinophage has been isolated from greenhouse soil; it proved specific for cultures of *S. olivaceus*. It was suggested to use this actinophage for the identification of cultures of this species. Out of 17 *S. olivaceus* cultures tested, 9, or 53 per cent, proved lysogenic. The actinophages isolated from these cultures proved identical to one another and somewhat different in their lytic properties from the phage isolated from the soil.

Sveshnikova and Pariskaya made a detailed examination of the occurrence of actin-

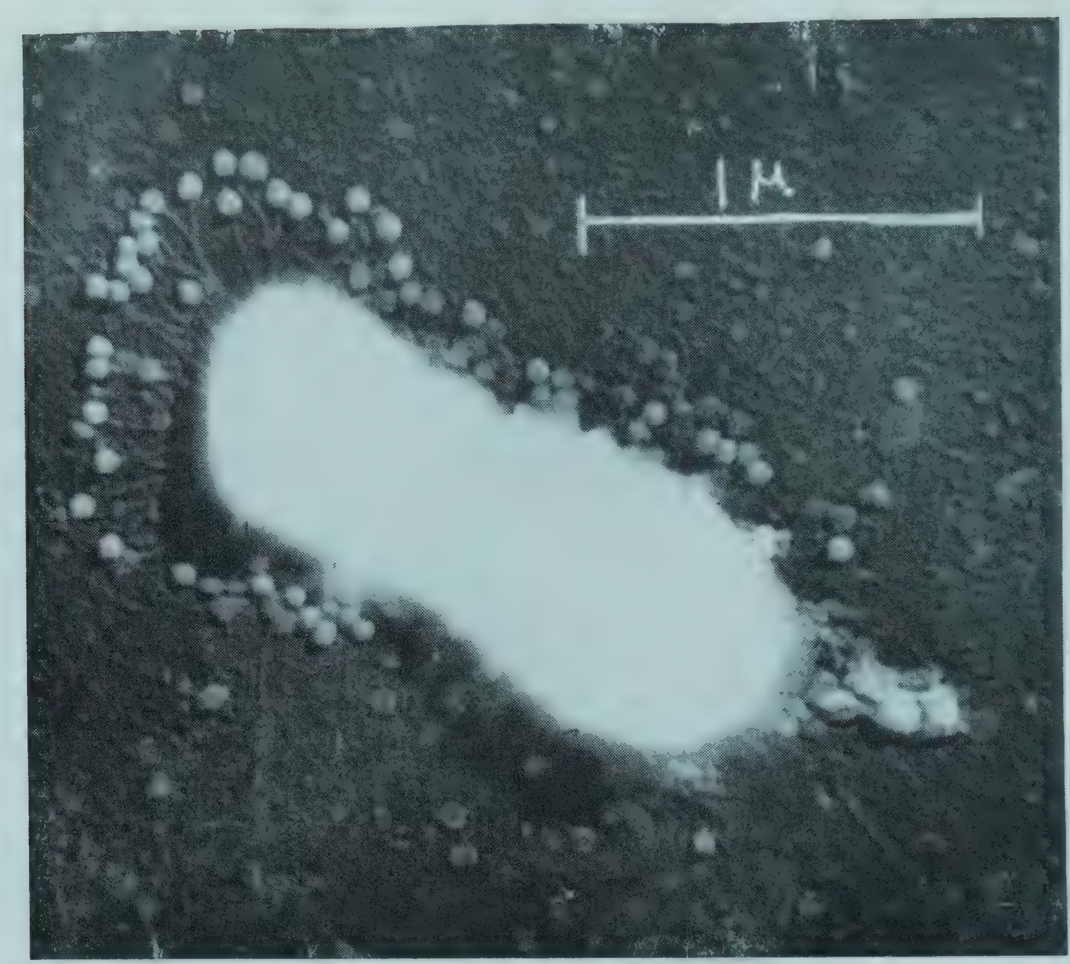


Figure 77. Phage particles adsorbed on a streptomyces spore (Reproduced from: Mach, F. Centr. Bakteriol. Parasitenk. Abt. II; 111: 556, 1958).

ophage in greenhouse soils. In four out of fourteen samples of soil, free actinophages were revealed by direct count. In most cases these actinophages proved to be polyvalent. In another ten samples of greenhouse soils and in twenty-five samples of forest and meadow soils, as well as in filtrates of field soils, no free actinophage was found. Many actinomycetes isolated from these soils were susceptible to the eight different actinophages previously isolated. Mach (1958) isolated from composts and forest litter three actinophages of which two were polyvalent and one strongly specific. These three phages were morphologically distinct from one another, as shown by the electron microscope.

Bradley and Anderson (1958) isolated from soil three streptomyces phages and five nocardia phages. Of the former, one attacked a culture designated as *N. paraguayensis*, and of the latter, three attacked streptomyces. All other Actinomycetales were resistant to both types of phage, as shown in Table 45. On the basis of these results, the authors concluded that the genera *Nocardia* and *Streptomyces* are closely related and should not be separated into different families. Such generalization is hardly justified. The *N. paraguayensis* used in these tests is not a nocardia, but a streptomyces, as will be brought out in Vol. II. The nocardia phages that attacked the streptomyces cultures may have been polyvalent phages, a potentiality indicated in the early studies of Wieringa and Weibols (1936). The sensitivity to phages as a criterion for species and varietal characterization of organisms will be discussed in detail in Vol. II.

The inhibition of the phage by chemical agents was studied by Perlman *et al.* (1951). Gause *et al.* (1957) reported that actinomycetes possess the ability to produce antibiotics which defend themselves against actinophage action. It was said that certain substances may defend actinomycetes against lysis by phages and simultaneously display a protective action toward other actinomycetes. Among 1,000 cultures studied, about one-half displayed the ability to hinder actinophage activity, such ability being found both among cultures hindering bacterial growth and among cultures lacking antibacterial action.

TABLE 45

Effect of several actinophages upon different Actinomycetales (Bradley and Anderson)

Lysis of a host by a standard phage suspension is indicated by a plus sign; no lysis is denoted by a minus sign.

Hosts	Actinophages					
	SP-3	SP-4	SP-8	NP-3	NP-4	NP-5
S. griseus S34	−	+	−	+	−	−
S. griseus S104	+	+	+	+	+	+
S. griseus 1945	−	−	+	+	+	−
S. olivaceus S11	+	+	+	+	+	−
S. venezuelae S13	+	+	+	+	+	+
S. cyaneus S45	−	−	−	+	−	−
Nocardia sp. 3403	−	−	−	−	−	+
*N. paraguayensis**	+	+	+	+	−	−
N. madurae	−	−	−	−	+	−
N. brasiliensis	−	−	−	−	−	−
N. asteroides	−	−	−	−	−	−
Actinoplanes sp.	−	−	−	−	−	−
Streptosporangium sp.	−	−	−	−	−	−
Mycobacterium phlei	−	−	−	−	−	−
Micromonospora sp.	−	−	−	−	−	−

* This culture is actually a streptomyces.

Hemolysin Production

Waksman (1919) demonstrated that the property of bringing about hemolysis of red blood cells is widespread among actinomycetes. This property was found to vary quantitatively for different organisms. A comparative study of the hemolytic properties of certain pathogenic forms (Waksman, 1918) brought out the fact that hemolysis of blood in blood agar, liquefaction of blood serum, clotting and subsequent peptonization of milk, and liquefaction of gelatin, all run parallel.

Lieske (1921) made a comprehensive study of hemolysin formation by actinomycetes obtained from various sources. There was no correlation between this property and the ability of the organisms to liquefy gelatin or to dissolve coagulated egg-albumin. The hemolytic action of actinomycetes was found to be an extracellular phenomenon, occurring during the very early growth of the organisms. An active preparation could be obtained by growing the organisms on blood agar plates, extracting the agar with salt solution, and filtering. Such extracts were capable of rapidly dissolving blood suspended in salt solution. The hemolytic activity of the extract was not destroyed by boiling; on the contrary, it was increased by such treatment. It was lost, however, on heating at 120°C in the autoclave. In this respect, it was similar to the hemolysis of certain gram-negative bacteria. This phenomenon suggests that the active substance is not truly enzymatic in nature. No antihemolysin could be demonstrated in the normal blood or in a patient suffering from actinomycosis.

Lieske reached the conclusion that the hemolysin effect was purely accidental and was caused by certain nonenzymatic normal metabolic products of the organisms. He also emphasized the lack of correlation between hemolysin production of microorganisms and their pathogenicity. Pathogenic organisms, including freshly isolated anaerobes and *N. farcinica*, were nonhemolytic, whereas many of the saprophytes were strongly hemolytic. The hemolytic property was changeable in nature; freshly isolated, nonhemolysing cultures can be made strongly hemolytic on repeated transfer upon blood agar media.

CHAPTER 11

Production of Enzymes

Actinomycetes produce a variety of extracellular and endocellular enzymes. Some of these enzymes have been isolated from the culture filtrates or the mycelium, concentrated, and purified. Others have only been demonstrated in the mycelium of the organism.

Lysozyme

The production of lysozyme systems by actinomycetes at first aroused considerable attention. These systems were confused, however, with autolytic and bacteriolytic mechanisms, on the one hand, and with antibiotics, on the other.

In his classical studies of the lytic agents of *B. subtilis*, Nicolle first suggested that bacteriolytic substances produced by microorganisms might have properties in common with enzyme systems. According to Welsch (1947), the bacteriolytic system of certain actinomycetes is lysozyme-like in nature and is able to digest bacterial cell wall substrates. However, according to Ghuysen and Salton and Ghuysen, such enzyme systems, unlike lysozyme, are able to liberate amino acids but not reducing substances.

Salton defined the enzymic properties of lysozyme on the basis of the following determinations: (a) turbidity reduction of isolated cell wall structures or lysis where the wall is *in situ* as with intact bacterial cells; (b) liberation of reducing groups; (c) liberation of an acetylamino sugar complex of glucosamine and the acidic hexosamine. Such enzymes as the actinomycetin complex possess only the ability to dissolve isolated bacterial cell walls; they are wrongly classified, therefore, with lysozyme. The ability of purified enzymes isolated from the actinomycetin complex to form amino acids sets them aside from lysozyme, which forms reducing groups.

The assertion of Kriss that actinomycetes produce "lysozyme" still requires confirmation of the ability of these organisms to form enzymes that liberate reducing groups and an amino sugar complex of the type released by lysozyme as well as the peptidase type, in accordance with the requirements laid down by Salton.

Proteases

Münter, Waksman, and Lieske first established that various actinomycetes, mostly members of the genus *Streptomyces*, possess strong proteolytic activities. Some cultures were able to decompose very energetically proteins in gelatin, egg-white, and blood serum. This is true of both saprophytic and pathogenic types. They vary greatly, in this respect, both qualitatively and quantitatively, as can be simply demonstrated by the process of gelatin liquefaction or casein decomposition in ordinary plates. As a rule, species of *Nocardia* are poorly proteolytic, whereas certain species of *Streptomyces* are highly active in this respect. The degree and rapidity of proteolysis also vary with individual species.

Stapp found that out of 477 freshly isolated cultures of streptomyces, only one failed to liquefy gelatin. The liquefying action of the others was characterized by varying degrees of rapidity. Many of the organisms produce a soluble brown pigment in gelatin, which, according to Beijerinck, is in the nature of a quinone that tends to harden the liquefied portion of the gelatin.

The quantitative ability to secrete proteolytic enzymes can also be measured by the degree of gelatin liquefaction and of casein hydrolysis. Many species are also able to decompose complex vegetable and animal proteins. Culture filtrates of certain actinomycetes were found to contain at least two proteolytic enzymes, one capable of digesting casein and the other of attacking the proteins of bacterial cells.

According to Chaloupka, cultures of streptomyces cultivated under different conditions secrete more protease in an environment with a low concentration of nitrogen than in media rich in nitrogen. A decrease in the concentration of sugars in the medium brings about a decrease in the secretion of the enzyme. Secretion of protease depends on the form of nitrogen, and is lowest in a protein medium, higher with lower peptides, and highest in media containing complex peptides and amino acids. Low secretion of the enzyme in protein media is accompanied by vigorous submerged sporulation of streptomycetes; high secretion is connected with lysis of the mycelium. Growth of the culture and enzyme production are greatly stimulated by potassium ions.

The proteolytic enzymes of actinomycetes are more resistant to the effect of higher temperatures than are corresponding animal enzymes; the former enzymes are able to withstand heating at 70°C for 30 minutes though at 80°C they are destroyed. Lieske found that the resistance of the enzymes to temperatures is greater than that of the living cells of the organisms, which are killed at 62 to 65°C. According to Krassilnikov (1938), many cultures are destroyed upon being heated at 40 to 45°C for a long time, but their proteolytic capacity is not affected.

The proteolytic activities of the various species of actinomycetes are so marked that Waksman (1919) suggested the use of this property for diagnostic purposes. Lieske, however, stated that proteolysis is not a constant property and cannot be used for characterization of the organisms. Krassilnikov (1938) tested 200 cultures every 8 to 12 months for 3 to 5 years. Various forms of gelatin were used for the test. The results were always identical. A strain that dissolved gelatin rapidly when first isolated continued to do so after 1, 2, 3, 4, and 5 years. Strains that failed to liquefy gelatin at first failed to do so after 2 to 5 years' cultivation. The nonpigmented forms were most active. The pigmented forms were least active.

Proteolysis may occur only at a late stage in the development of the organism. This may be due to the formation of endoenzymes, which are liberated on the death of the cells, as contrasted with the exoenzyme produced at an early stage of the development of the mycelium. The diagnostic properties of proteolysis must, therefore, be based upon early observations during the stage of the rapid growth of the organisms. McConnell (1950) and Dion (1950) studied the extracellular proteases produced in submerged culture.

Species of *Nocardia*, as a rule, possess much weaker proteolytic systems than do *Streptomyces* species. Some, like the pathogenic *N. asteroides* and the saprophytic *N. ruber* and *N. viridis*, do not liquefy gelatin at all. Some of the yellow species (*N. flava*) are weak liquefiers. There are also reports in the literature that pigmented nocardias did not liquefy gelatin. The white (*N. alba*) forms, however, are able to liquefy gelatin.

No large scale production of proteolytic enzyme preparations has so far been obtained from actinomycetes. Sterile culture filtrates of certain species were found to exert a marked effect not only upon animal proteins but also upon proteins derived from soybeans, peanut meal, and corn meal. According to Simon, *S. griseus* produced protease in a medium containing 2 per cent soybean meal. An active enzyme preparation with a potency equal to that of pancreatin was obtained; the activity did not decrease on dialysis. Casein, soybean, protein, fibrin, and peptone could be used as substrates. The optimum reaction for the activity of the enzyme was pH 8.2. An aqueous solution of the enzyme was inactivated at 60°C in 30 minutes. Further studies on the production of proteolytic enzymes by various actinomycetes have been made by Naeslund and Dernby (1923). The formation by different streptomycetes of proteolytic enzymes as well as of amylolytic and inverting systems has been discussed in detail by Jensen (Table 46). See also Tytell *et al.* (1954).

TABLE 46

Production of diastase, invertase, and protease by different actinomycetes (Jensen, 1930)

Organism	Starch hydrolysis	Sucrose inversion	Proteolysis
S. griseus, 5 strains	0	0	+++
S. griseoflavus, 2 strains	0	0	+++
S. cellulosae, 5 strains	0	0	++
S. fulvissimus, 3 strains	0	+	++
S. olivaceus, 5 strains	0	0	++
S. violaceus-ruber, 3 strains	+	0	++
S. diastatochromogenus, 3 strains	0 to +	+++	+
S. bobiliae, 2 strains	++	+++	+
S. halstedii (?), 2 strains	0 to +	–	–
S. aureus, 2 strains	+++	0	+
S. pheochromogenus	+++	0	+
S. erythrochromogenus	+	–	+

Bechtereva *et al.* (1958) studied the course of accumulation of active proteolytic enzymes by *S. violaceus* and *S. lavendulae*. The period of intensive accumulation of active proteolytic enzymes in a simple synthetic medium and in a corn-extract medium was found to be related to the decomposition of the cells. Upon submerged fermentation in media containing proteins, the release of active proteolytic enzymes may accompany not only decomposition of mature cells but also vigorous growth of the young healthy hyphae. The concentration of the nitrogenous components in the medium greatly influences the rate of decomposition of the *S. lavendulae* mycelium and the accumulation of active proteolytic enzymes.

Rennet or Lab

Coagulation of milk by microorganisms can be brought about either through the action of the lactic acid formed from the lactose or by means of an enzyme, usually designated as lab or rennet. Since the majority of actinomycetes do not form any lactic acid from lactose, the production of lab can easily be established. The addition of some $CaCl_2$ is favorable to the coagulation process. While the optimum temperature for growth of the organism may be 28 or 37°C, that for enzyme action is 55 to 65°C. Heating to 70°C has no injurious effect, but activity is destroyed at 80°C for 30 minutes. Lieske obtained an active preparation of lab by precipitating a liquefied milk culture of the organism with alcohol. The lab enzyme is also produced by active cultures grown in other media, such as blood serum.

The fact that certain actinomycetes were capable of clarifying the milk without previous coagulation, whereas others brought about coagulation followed by varying degrees of decomposition of the coagulum, suggests the possibility that lab is an enzyme distinct from the true proteolytic enzymes (Waksman, 1918).

Keratinase

Noval and Nickerson obtained, from a culture of *S. fradiae*, a highly potent preparation of keratinase that digested hoofmeal, wool, and feathers. Noval (1957) made a comprehensive study of this preparation.

Three strains of *S. fradiae* were isolated and found capable of rapidly solubilizing 80 to 90 per cent of native keratin. One of these, *S. fradiae* 3739, was isolated as the most active keratin-digesting strain and was used for the preparation of the enzyme. Significant stimulation in the digestion of wool by this culture was obtained by increasing the Ca^{++} and/or Mg^{++} concentration of the media. Approximately two-thirds of the cystine of the digested wool accumulated as soluble sulfhydryl compounds in the culture broth during the digestion. The sulfhydryl material was very stable to aeration, heating, and acidification but was substantially destroyed by addition of organic solvents to the acidified broth in the presence of light. Neither cysteine nor sulfide was detectable in the culture broth during or after the active digestion of wool. Most (75 per cent) of the nitrogen of the solubilized wool was accumulated in the form of ammonia.

The cell-free culture broth of *S. fradiae* 3739 was capable of enzymatically digesting keratins and casein. The enzymes that caused both of these digestions were similar in their optimal activity at about pH 9 and in their nonsensitivity to sulfhydryl reagents. Magnesium appeared to be the metal required for the digestion of wool by the culture broths.

The culture broths of *S. fradiae* were capable of solubilizing a maximum of 10 to 20 per cent of several native keratinaceous substances; trypsin and papain could solubilize, at the most, about half as much (5 to 10 per cent) of each of the same keratins. By ammonium sulfate precipitation, a product was obtained that had about 16 times as much wool-digesting activity per milligram of protein as did the culture broth.

Urease

Various actinomycetes, like *S. griseus*, were found (Simon) to produce urease. This enzyme was found also in cultures grown in urea-free media; hence it is not adaptive in nature. It was suggested that urea may be produced by the organism from guanidine by the action of guanidase.

Deguanidase

S. griseus was found by Roche *et al.* to produce a system of deguanidases that are active at pH 7.5 upon different monosubstituted guanidines. This system comprises a mixture of enzymes different from arginase. Its diffusion in the medium can bring about the destruction of streptomycin.

Chitinase

Nearly all streptomyces are capable of producing an enzyme that has the capacity to hydrolyze chitin. Jeuniaux reported that this enzyme is formed in a simple synthetic medium containing chitin as the only source of carbon and nitrogen. The enzyme is also produced in the absence of chitin; the presence of chitin in the medium was not essential for, although it favored, formation of the enzyme. The presence of glucose tended to repress the formation of chitinase. The enzyme was found to be rather unstable in culture filtrates, but the presence of chitin tended to stabilize it.

Bucherer has shown that various species of *Streptomyces*, notably *S. griseolus*, *S. exfoliatus*, *S. fradiae*, *S. aureus*, and *S. griseus* are able to break down chitin. According to Schmidt-Lange and Bucherer, both pathogenic and saprophytic actinomycetes are capable of producing the enzyme chitinase.

Yamaguchi (1957) found that different species of streptomyces, such as *S. fradiae*, have the capacity to produce a powerful cuticle (of pig *Ascaris*) digestive substance that could be precipitated from culture filtrates and by ethyl alcohol, acetone, and other protein-precipitating agents. The enzyme-like substance was distinct from the casein-digesting agent.

Amylases

Numerous actinomycetes are able to hydrolyze starch rapidly, either to the dextrin stage or directly to maltose and glucose. The production of amylolytic enzymes by actinomycetes was first recorded by Fermi, who found most of the actinomycetes tested capable of producing such enzymes. These results were later confirmed by various investigators, including Caminiti. Some claimed that the starch is hydrolyzed only partially, not to the sugar stage.

Krainsky made a detailed study of a large number of actinomycetes that were found capable of producing amylase. This phenomenon was further studied extensively by Waksman and Lieske, who observed that only very few actinomycetes lack the ability to produce such enzymes.

For the screening of a large number of cultures of actinomycetes, agar media containing starch as the source of carbon are used. The plates are streaked and allowed to incubate. After 5, 10, 15, and 20 days, the surface of the agar is covered with a solution of I-KI, and the amount of starch hydrolyzed is measured by the width of the clear zone around the streak. Formation of zones 1.0 to 1.5 cm wide after 10 days' incubation is an index of good amylase production. For the production of amylolytic enzymes, inorganic sources of nitrogen, especially nitrates, appear to be preferable to organic compounds.

Stapp recorded that 83 per cent of all actinomycetes isolated from soil and belonging to the genus *Streptomyces* produced amylase. Although the amylolytic property is characteristic of the species, as noted by Krassilnikov, the wide distribution of this property tends to reduce its diagnostic value. A single alpha type amylase was found to be produced by five different streptomyces species (Simpson and McCoy, 1953). The mechanism of breakdown of starches by actinomycetes is discussed further by Bois and Savary (1945).

The amylases of actinomycetes are able to withstand the effect of higher temperatures better than are the cells of the organisms producing them. Surovaya obtained a potent diastatic preparation from a culture of *S. diastaticus*. The organism was grown on a potato medium, and a satisfactory enzyme preparation, designated as "superbiolase," was obtained. This preparation was active at 70 to 100°C and had an optimum pH at 6.6 to 6.7. The starch was converted first into soluble form and then into dextrin. Saccharification of the dextrin proceeded much more slowly than the liquefaction of starch.

The hydrolysis of mannosidostreptomycin to streptomycin, by various strains of *S. griseus*, is said to be due to an amylase (Christensen *et al.*, Langlykke and Perlman). Maruta and Tanaka isolated the enzyme mannosidostreptomycinase by precipitation of the culture broth with 1 to 2 per cent lead acetate. The optimum pH for the action of the enzyme was 6.8 to 7.4 and optimum temperature 37°C. Certain aldohexoses, such as glucose and mannose, inhibited the action of the enzyme; D-sorbitol produced an accelerative effect. In the regular streptomycin fermentation by *S. griseus*, the content of mannosidostreptomycin is high during the early stages of fermentation and low in the older stages. The presence of glucose in the early stages represses the action of the enzyme.

Many actinomycetes are able to attack dextrins, glycogen, and inulin and to produce the corresponding enzymes. Lieske, who was one of the few to study these enzyme systems, reported that the anaerobic pathogenic forms, or species of *Actinomyces*, differ from the aerobic forms in that they form such enzymes in mere traces, if at all. No attempt has been made to study these enzyme systems in detail or to utilize them for any practical purposes.

Polysaccharidases

Various actinomycetes are capable of utilizing agar and other polyuronides as sources of energy. The agar is thereby liquefied. This is true particularly of such forms as *S. coelicolor* (Stanier).

A detailed study of the enzymes involved in the decomposition of seaweeds and seaweed products (laminarin and alginates) by actinomycetes has been made by Chesters *et al.* (1956). Various nocardias (*N. citrea*) have been found to be active in breaking down calcium alginate and laminarin. Certain streptomycetes were particularly active in decomposing laminarin. In this respect, they were much more active than bacteria. The enzymes laminarinase and alginase were isolated from cultures of these organisms and found to be highly active upon the corresponding substrates, as well as upon starch and calcium pectate. These enzymes were obtained either from the culture filtrates of the organisms or by treating the mycelium with 15 per cent ethyl alcohol.

Sorensen (1957) found that both *S. albus* and *M. chalcea*, when grown in a xylan-containing medium, have the capacity to produce the enzyme xylanase. This enzyme is extracellular and can attack the xylan chain at random along its length, yielding a mixture of shorter or longer chain fragments. These saccharides, of which xylotriose is the shortest, are attacked further by xylanase, giving the following end products: xylose, arabinose, xylobiose, and uronic acid or uronic acid-xylose oligosaccharides. The xylanase produced by the streptomyces contained two fractions, of which one traveled toward the anode and the other remained at the starting point. The two fractions were found to represent two proteins with identical enzymatic functions.

Invertase

Invertase is widely produced by actinomycetes, as shown by Caminiti, Krainsky, and Waksman. Lieske was unable to demonstrate the production of this enzyme by the cultures he investigated, but he did not deny such capacity. The formation of invertase and other saccharidases by actinomycetes has also been studied by Hofmann and Latzko (1950).

The ability of some actinomycetes to utilize sucrose as a source of carbon is largely dependent upon the property of the organisms to produce invertase. This capacity has not been established for all organisms, however. Krassilnikov says that nocardias are able to utilize sucrose without prior inversion. According to Waksman, only those forms that are able to produce invertase make abundant growth on media containing sucrose.

In view of the constancy of this property, it has been suggested that invertase production be utilized for species differentiation.

Cellulolytic Enzymes

Although the property of decomposing cellulose is widely distributed among microorganisms, neither the cellulolytic mechanisms nor the cellulases involved in the processes of decomposition are well understood. As shown previously, many actinomycetes, especially streptomyces, are able to grow on cellulose as the only source of carbon. This was established through the work of Fousek, Krainsky, Waksman, and others. Various actinomycetes capable of decomposing cellulose have been described under different names, such as *Mycococcus cytophagus* of Bokor, *Micrococcus cytophagus* of Merker, and the organism described by

Krassilnikov as *Proactinomyces cytophagus*, all of which, especially the first, appear to belong to the nocardia group.

There is no question that cellulolytic enzymes are involved in these processes, although they have not yet been demonstrated with any degree of certainty.

Lipase and Esterase

Actinomycetes are found abundantly on fats, especially on butter. Jensen demonstrated in 1902 that certain chromogenic actinomycetes are able to grow in sterile butter and produce considerable amounts of acid. Lieske reported that actinomycetes, especially the aerobic forms producing long mycelium, are capable of attacking a variety of fats. The fatty acids produced are neutralized by the salts in the medium, giving rise to characteristic, mostly needle-shaped, crystals. To what extent hydrolytic mechanisms of the lipase and esterase type are involved is still uncertain. Some of these systems appear to play an important part in the spoilage of various fats and cacao and in odor production.

Oxidative Enzymes

Actinomycetes possess a number of oxidative mechanisms, only a few of which are recognized at the present time (Sano, 1902). The production of phenol oxidases by certain actinomycetes has recently attracted considerable attention. Hockenhull *et al.* (1954) studied α-phenyl mannosidase production by *S. griseus*. After a suitable manometric method for the determination of this enzyme had been worked out, a phenol oxidase of the laccase type was found by Sevcik in *S. antibioticus*. This laccase was an endoenzyme with a maximum activity at pH 4.0 to 4.5. Hydroquinone was oxidized most rapidly, catechol more slowly, and *p*-phenylenediamine very slowly. When growing *S. antibioticus* in submerged cultures on different media using a rotating shaker, Sevcik found a direct relation between the production of the antibiotic actinomycin and that of phenol oxidases. On the basis of these results, he concluded that phenol oxidase of the laccase type participates most probably in the biosynthesis of actinomycin by *S. antibioticus*.

Küster demonstrated the presence of phenol oxidases in the autolyzate of various cultures of streptomyces such as *S. viridochromogenes*. These enzymes are capable of producing humic acid-like substances; this finding was believed to be evidence of the role of actinomycetes in humus formation.

Hirsch and Wallace (1951) studied the octanoxidase system of *S. aureofaciens*, and Kempf and Sayles (1946) the oxidation-reduction potentials of *S. griseus*. Cochrane made a comprehensive examination of the enzymes involved in the utilization of carbohydrate by *S. coelicolor*. The organism forms the enzymes phosphofructokinase, aldolase, triose phosphate isomerase, triose phosphate dehydrogenase, phosphoglyceryl kinase, enolase, and ethanol dehydrogenase. Growth of *S. coelicolor* resembles yeast fermentation in its requirements for phosphate,

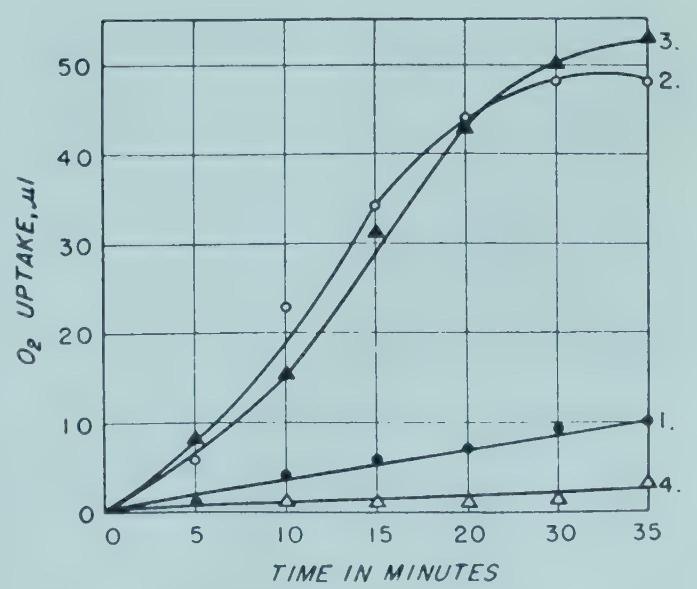


FIGURE 78. Oxidation processes by cell-free extracts of *N. corallina*: 1. endogenous; 2, thymine; 3. uracil; 4. barbiturate. (Reproduced from: Lara, F. J. S. J. Bacteriol. **64**, 281, 1952).

adenosine diphosphate, and diphosphopyridine nucleotide, as well as in its susceptibility to iodoacetate and fluoride in the breakdown of fructose 1,6-diphosphate.

Intact cells or extracts were unable to ferment hexoses under normal conditions. It was suggested that obligate aerobiosis of *S. coelicolor* results from a biochemical lesion, the inability to regenerate anaerobically the diphosphopyridine nucleotide reduced in the oxidation of triose phosphate.

According to Sato, aerobic actinomycetes possess the respiratory pigments of the *a*, *b*, *c*, *d*, and d_2 cytochromes. On the other hand, the anaerobic actinomyces of the alkali type possesses *a*, *b*, *d* cytochromes, and those of the acid type possess no cytochrome at all. Birk *et al.* demonstrated the production of a *b*-type cytochrome by *S. fradiae.*

In the presence of 2,6-dichloroindophenol, crude extracts of *S. scabies* were found by Douglas and San Clemente capable of catalyzing the dehydrogenation of succinate, citrate, malate, and glutamate. Transaminase activity was also demonstrated, since α-ketoglutarate was converted to glutamate in the presence of aspartate, leucine, or valine as amino group donors.

According to Inoue (1958), *S. griseus* possesses the oxidase of the intermediates in the Krebs cycles. Glucose, pyruvate, acetate, oxalsuccinate, and lactate were oxidized readily, but the oxidation rate of citrate was very low in the given condition. Formate was not oxidized at all. Glucose oxidation was inhibited by monoiodoacetate and sodium fluoride. Succinate oxidation was inhibited by malonate, the latter being oxidized slightly. Citrate appeared to be formed as a result of an oxalacetate-acetate condensation reaction, a reaction not inhibited by streptomycin.

Inoue further reported that *S. griseus* possesses cytochromes *a*, *b*, and *c*; their wave lengths were found at 600 mμ, 567 mμ, and 550 mμ, respectively. Cyanide inhibited oxidation of acetate, malate, and lactate; glucose, pyruvate, oxalacetate, succinate, and oxalsuccinate were inhibited only slightly. Sodium azide oxalacetate slightly inhibited oxidation of succinate and lactate, but not glucose and pyruvate. It was suggested that the cytochrome system acts as an electron transfer system in *S. griseus*; the participation of some other systems, such as that of flavoprotein, may also be considered.

According to Musilek and Sevcik (1958), the addition of sodium arsenite in a final concentration of 4×10^{-4} *M* to the medium of *S. erythreus* reduced biosynthesis of erythromycin by 87 per cent, with a simultaneous increase in pyruvic acid. Sodium acetate and sodium propionate in final concentrations of 0.5 per cent decreased the inhibitory effect of arsenite on erythromycin biosynthesis. Other salts of organic acids did not reduce the effect of arsenite. The latter completely inhibited oxidative decarboxylation of pyruvate and oxidation of acetate by the washed mycelium of *S. erythreus*, but only partly inhibited glucose oxidation. Biosynthesis of erythromycin depends on uninterrupted oxidative decarboxylation of pyruvic acid to acetic acid. The authors suggested the probability of the part played by acetic acid as the initial substrate in the biosynthesis of propionic acid, which is assumed to be the precursor of the lactone nucleus in the erythromycin molecule.

Catalase

The relation between the development and catalase activity of *S. griseus* was studied by Kovacs and Matkovics. A close relation was found between streptomycin production and catalase activity. A high catalase activity was not necessarily a prerequisite for streptomycin production, but was always present with high yields of streptomycin. With only little catalase activity, there was very little streptomycin produced.

Penicillinase

The ability of actinomycetes to produce penicillinase, the enzyme capable of oxidizing penicillin, is widely distributed. Streptomycetes are in general resistant to penicillin; there is no relation, however, between penicillin-resistance and formation of penicillinase. This enzyme is heat-labile. It can be concentrated by precipitation with ammonium sulfate and acetone, a response that suggests its protein nature (Welsch).

Tyrosinase

The production of a brown pigment by actinomycetes grown on protein media has usually been associated with the ability of the organisms to form tyrosinase. According to Beijerinck, there is involved in the reaction the formation of a quinone, which turns brown at an alkaline reaction and in the presence of oxygen. The action of quinone in the presence of iron was found to be similar to that of the enzyme tyrosinase. Since an excess of oxygen is required for the formation of the quinone, only limited amounts are found in deep cultures. The quinone is believed to be formed from the peptone in the medium; although good growth was produced on media containing asparagine, KNO_3, and ammonium sulfate as sources of nitrogen, only traces of quinone, if any, were found. The tyrosinase reaction is not involved in the production of all black pigments by actinomycetes; some species produce such pigments in purely synthetic media in the complete absence of peptone.

It has always been assumed that the potato scab organism is a chromogene, and that the formation of the black pigment is due to the tyrosinase reaction. Millard and Burr reported, however, that some of the plant pathogens did not give the tyrosinase reaction. Afanassiev could not confirm the pathogenicity of these cultures. Skinner concluded that the production of a dark color by the chromogenic actinomycetes is due to tyrosine metabolism.

Gregory and Vaisey found that natural and x-ray-induced mutants of *S. scabies* were tyrosinase-deficient and did not produce a brown ring in skim milk. All tyrosinase-positive cultures produced the brown ring. The tyrosinase-deficient cultures were virulent for potatoes. This fact demonstrates that there is no connection between pathogenicity and the tyrosinase reaction.

Steroid Oxidation

The ability of various actinomycetes to oxidize steroid hormones has recently attracted considerable attention, as shown in Chapter 9. "Resting cells" of certain streptomycetes are able to transform steroids. According to Turfitt (1944), various species of *Nocardia* are capable of attacking steroids, with the possible exception of halogen-substituted derivatives. The oxidation of cholesterol results in the formation of a cholesterone, followed by molecular fission, the products of which may be utilized by the organisms for their further growth.

Perlman *et al.* (1957) studied the enzymes

TABLE 47

Aryl sulfatase activity of rapidly growing mycobacteria, nocardias, streptomyces and corynebacteria (Wayne, Juarez, and Nichols)

Species	Number of strains tested	Number of strains positive
Mycobacteria		
M. fortuitum	15	15
M. phlei	5	5
M. smegmatis	4	4
M. rhodochrous	10	0
Miscellaneous species	29	29
Nocardias		
N. asteroides	56	13
N. brasiliensis	4	1
N. madurae	6	1
Miscellaneous species	33	6
Streptomyces	8	0
Corynebacteria	6	0

that oxidize progesterone. Schiesser studied cortisone-oxidizing enzymes. Harris *et al.* (1957) found that *S. globisporus*, *S. viridochromogenes*, and other streptomycetes are capable of deacetylation and oxidation of dehydroepiandrosterone acetate.

A detailed discussion of the literature on steroid oxidation by actinomycetes is found in the reviews of Eppstein *et al.* and Wettstein (1955).

Other Enzymes

Numerous other enzymes and coenzymes have been found to be produced by actinomycetes. It is sufficient to mention coenzyme A (Gregory *et al.*, 1952). The ability of various nocardias (*N. asteroides*, *N. pelletieri*) to bring about the demyelinization of bovine spinal cord may be due to an enzyme system. Some of these mechanisms produced by *Streptomyces* and *Nocardia* species are thermostable and others are thermolabile (Adelson *et al.*). Anaerobic actinomycetes are able to produce acid phosphatase (Howell and Fitzgerald, 1953).

Wayne *et al.* developed aryl sulfatase tests for differentiating saprophytic mycobacteria from the tuberculosis organisms. This study was of particular interest in differentiating atypical mycobacteria and nocardias. Only 21 per cent of all nocardias tested and none of the streptomycetes or corynebacteria produced the enzyme (Table 47). Almost all mycobacteria hydrolyzed demonstrable amounts of phenolphthalein disulfate if sufficient inoculum was used and permeability differences compensated for. The interesting conclusion was reached that if *M. rhodochrous* is a true mycobacterium, it is the only member tested which does not produce aryl sulfatase.

The production of enzymes of the isocitritase pathway was demonstrated by Bardi *et al.* (1958) for *N. rugosa*.

Lara (1952) reported that enzyme preparations of the *N. corallina* group can be obtained by extracting alumina ground cells. The activity of these extracts against thymine and uracil was demonstrated only when methylene blue was added. These compounds were oxidized to substances having the over-all composition of 5-methyl barbituric acid and barbituric acid, respectively. In one experiment uracil was formed from thymine; the results of many other experiments, however, indicated that the demethylation of the latter did not normally proceed under the conditions used, leading to the conclusion that normally uracil is not an intermediate product in the decomposition of thymine. Cell-free extracts of *N. corallina* with activity towards thymine, uracil, and barbituric acid were obtained only from cells grown in either thymine, uracil, or barbituric acid; enzyme preparations from glucose grown cells were devoid of such activity. This points to the adaptive nature of the enzyme system (Fig. 78).

Barbituric acid was hydrolyzed by the enzyme preparation with the formation of 1 mole of CO_2, 2 moles of NH_3, and 1 mole of malonic acid, for a pathway of thymine and uracil degradation according to the following scheme:

$$\begin{tikzcd}
\text{Thymine} \arrow[r] \arrow[d, "?"] & \text{5-Methyl barbituric acid} \arrow[d, "?"] & & \\
\text{Uracil} \arrow[r] & \text{Barbituric acid} \arrow[r] & \text{Malonic acid} + \text{Urea} \arrow[dl] \arrow[d] & \\
& CO_2 & NH_3 &
\end{tikzcd}$$

CHAPTER 12

Production of Vitamins and Other Growth Factors

Various actinomycetes and their metabolic products exert a favorable effect upon the growth of lower forms of life, including fungi and other microorganisms, and upon higher forms of animal and perhaps plant life. This is due, partly at least, to their ability to synthesize several vitamins.

Herrick and Alexopoulos grew *S. viridochromogenes* in a liquid medium, then filtered the culture and autoclaved it. The fungus *Stereum gausapatum* inoculated upon this medium produced a heavier growth than usually obtained on sterile media. Even more striking results were obtained when *Phycomyces blakesleeanus* was used as the test organism; since this culture is used in assaying for the presence of thiamine, the conclusion was reached that actinomycetes produce this vitamin. In a further study of a number of cultures of actinomycetes, it was demonstrated that all were able to produce thiamine or its intermediate or precursor.

Mackinnon observed that various strains of *S. albus* have a marked stimulating effect upon the growth of *Trichophyton discoides*. This effect was comparable to that exerted by the presence of thiamine. The same effect was obtained when *P. blakesleeanus* was inoculated on a synthetic medium in which the streptomyces had previously been grown. Filtered cultures of *S. albus* added to synthetic media had the same effect upon the growth of thiamine-requiring organisms.

The formation by *S. griseus* of a growth factor for *Leuconostoc citrovorum* was demonstrated by Emery *et al.* (1950). This factor was not identical with either B_{12} or B_{13}. This factor had no activity upon pernicious anaemia, but possessed leucocyte-stimulating activity.

The production of carotenoids by actinomycetes has also been demonstrated. Protiva has shown that certain actinomycetes are able to produce riboflavin and flavoprotein in synthetic media.

Formation of Vitamin B_{12}

The announcement, in 1948, by Rickes *et al.* that certain actinomycetes are able to produce vitamin B_{12} resulted in much interest in the commercial possibilities of these organisms as sources of this vitamin. It has now been established that numerous actinomycetes are able to produce this vitamin if cobalt salts are added to the media to serve as precursors. The production of B_{12} can be measured by microbiological assays. Various strains of *S. griseus*, including the streptomycin- and grisein-producing forms, and various *Streptomyces* species, such as *S. fradiae* and *S. aureofaciens*, are able to produce some vitamin B_{12} without affecting the yields of the corresponding antibiotic substance. Certain nonantibiotic-producing actinomycetes, like *S. olivaceus*, were also found capable of producing this vitamin (Ganguly *et al.*).

According to Hall *et al.* (1953), *S. olivaceus* when grown under submerged aerobic conditions is capable of producing large amounts of vitamin B_{12}. The yields of the vitamin were influenced by the composition of the medium. In the presence of proteinaceous material such as distillers' solubles, glucose, $CaCO_3$, and cobaltous ion, about 1.5 μg of vitamin B_{12} was produced per millilter in deep tank fermentors, but as much as 3 μg of the vitamin was obtained in some media. Appreciable amounts of the B-complex vitamins, niacin, pantothenic acid, biotin, pyridoxine, thiamine, and riboflavin were produced.

By selective cultivation, including mutant formation by ultraviolet light and x-rays, it is possible to increase considerably the yields of vitamin B_{12} by a given culture.

The effect of cobalt as a limiting factor in the biosynthesis of the active vitamin B_{12} by *S. griseus* was immediately recognized (Hendlin and Ruger, 1950). Shull and Routien (1951), Pridham *et al.* (1951), Saunders *et al.* (1951), and Garey *et al.* (1951) made a survey of vitamin B_{12} production by different actinomycetes. Particular attention was paid to *S. griseus* (Rickes *et al.*, 1948) and *S. fradiae* (Jackson *et al.*, 1951).

Chemical investigations have brought out the fact that there are several forms of vitamin B_{12}. Possibly not all of them occur naturally but are formed during the isolation process. The incorporation of cobalt into the actinomycete metabolite has been established by the use of radioactive cobalt in fermentation media and the isolation of the vitamin containing the labeled isotope.

Some of the vitamin B_{12} produced by actinomycetes is bound on the cells, and may be released by treatment with acid, alkali, ionizable salts, sonic energy, or heat. Subsequent to release, the cell-free liquid may be treated with cyanide to convert the vitamin present to the more stable cyanide form.

In addition to vitamin B_{12}, various other biologically active compounds, designated as B_{12b}, B_{12c}, and B_{12d}, are produced by *S. griseus* (Anslow *et al.*). The formation of vitamin B_{12b} by *S. aureofaciens* was demonstrated by Lichtman *et al.* (1949). The B_{12b} was effective, by parenteral administration, in the treatment of Addisonian pernicious anemia.

In a study of the production and purification of vitamin B_{12} by various actinomycetes, Tarr has shown that B_{12a} was formed by *S. griseus* and by *S. aureofaciens* in aerated herring press water containing 2 mg per milliliter of added cobalt (as cobaltous nitrate). Highest recovery of vitamin B_{12a} in these products (about 1.1 mg per milliliter with *S. aureofaciens* and 0.8 mg per milliliter with *S. griseus*) was obtained by chromatography on filter paper strips treated with potassium dihydrogen phosphate, elution of the vitamin, and aseptic addition of the eluates to previously sterilized *Lactobacillus leichmannii* assay medium. Treatment of the crude fermentation products with potassium cyanide (2.5 mg. per milliliter) caused partial resolution of B_{12a} to B_{12}.

Letunova (1958) made a comprehensive study of cultures isolated from the lime of stagnant reservoirs of the biogeochemical province enriched in cobalt. Nearly 86 per cent of these cultures, as well as 90 per cent of strains isolated from lime deposits of the Co-impoverished province, were able to form vitamin B_{12}.

Certain nocardias are also capable of producing B_{12}. Bardi *et al.* (1958) studied a strain of *N. rugosa* that was found to release free porphyrins into the medium under suitable aeration conditions. By ultraviolet irradiation, mutants were obtained that gave a much higher yield of porphyrins. The relation between vitamin B_{12} and porphyrin production was demonstrated. The free porphyrins consisted mainly of coproporphyrin III, and of uroporphyrin, probably III, in minor amount. Traces of coproporphyrins I and II, of uroporphyrin I, and of other non-identified porphyrins were also released in the medium. The total free porphyrin content in broths may reach, in some strains, 20 to 40 μg/ml. The mutants producing greater amounts of porphyrins gave lower yields of vitamin B_{12} than the parent strain. Less aerated cultures produced more porphyrins and less vitamin B_{12}. The lack of cobalt lowered porphyrin production. Added δ-aminolaevulic acid (100 to 500 μg/ml) increased porphyrin production, but did not exert any effect on vitamin B_{12} levels. Small amounts of succinic and glyoxylic acids were detected in suspensions of *N. rugosa* incubated with citrate under anaerobic conditions. Since glyoxylic acid can be a precursor of glycine, it was suggested that this reaction might have some meaning in porphyrin biosynthesis by this strain.

A detailed discussion on the effect of cobalt concentration in the medium upon the production of vitamin B_{12} is given in Chapter 8. Darken (1953) reviewed the production of B_{12} by actinomycetes.

Other Vitamins and Vitamin-like Materials

Actinomycetes have also been found to produce various other porphyrin-like (Cortese) and iron-containing compounds, such as grisein. They also have the capacity to produce other water-soluble vitamins, notably coenzyme A, the pteroylglutamic acid derivative that promotes the growth of certain strains of *Leuconostoc citrovorum*.

Little is known of the mechanisms involved in the direct symbiosis of actinomycetes with insects, as in the case of the nymph of the hemipterous insect *Rhodnius prolixus*, the moulting and reproduction of which depend upon its infection with *N. rhodnii*. Brecher and Wigglesworth (1944) isolated a culture of a nocardia regularly from this insect reared in the laboratory. The microorganism is not transmitted through the egg but is taken up by the young nymph from the environment, such as the contaminated surface of the egg, and more often perhaps from the dry excreta of other members of the species. Insects were reared free from the actinomycete by sterilizing the surface of the egg and feeding with suitable precautions. They grew and moulted normally until the 4th or 5th instar. Moulting was then delayed or failed entirely in spite of repeated feedings of blood. Very few insects without the actinomycetes became adult, and those few were almost certainly incapable of reproduction. Normal growth and moulting and egg production were resumed when the insects were reinfected with the organism.

Little is also known of the role of the streptomycete that is able to infect nematodes that grow in the cockroach (Hoffman).

Other references in the literature concern the formation by certain actinomycetes of substances that exert a stimulating effect upon the growth of various organisms. It is sufficient to mention the work of Rehm on the presence in the mycelium of cultures of the *S. albus* group of substances that stimulate the growth of the fungus *Aspergillus niger*.

Growth Stimulating Effect of Antibiotics

The highly significant practical results obtained by the stimulating effects of antibiotics upon animal growth could not all be explained by the action of known vitamins. Moore *et al.* first observed, in 1946, this effect for an actinomycete antibiotic, namely streptothricin. At the present time, large quantities of the tetracyclines and streptomycin are employed in the feeding of nonherbivorous animals. Some of these growth factors are still unidentified (Fitz *et al.* 1956).

An early review of the effect of antibiotics upon the growth of swine has been presented by Brande *et al.*, and a detailed analysis of the nature of the growth promoting effect of antibiotics upon animals has been made by Porter (1957). Porter summarized the evidence that their use results in appreciable benefit to growing livestock. Early fears that their use for breeding stock might lead to the establishment of resistant strains of pathogenic bacteria have been found to be groundless. The feeding of antibiotics to animals was found to result in a healthier environment.

The mechanism by which the antibiotics produce this favorable effect on growth of the animals may be due to their action on the tissues, or primarily upon bacteria. This may depend partly on the state of health of the animals. Conditions of poor health can lead to a state of disease, making it difficult to draw a distinguishing line between the therapeutic effect of antibiotics and their nutritional action. Antibiotics may exert a direct antibacterial action on pathogenic bacteria in the gut or animal tissues. Their presence in small quantities in both the gut and tissues may also act as a prophylactic. Even in the absence of a state of disease there is a definite relationship between the effect of antibiotics on growth and the effect on the microbial flora. There is little or no evidence of consistent changes in the types and numbers of the microorganisms in the gut, possibly because present techniques are not sufficiently developed to detect changes in the gut population. Such changes may not take place and the antibiotics may act by altering the metabolism but not the morphology of the microorganisms. Porter postulated that such alteration may result either in an increased microbial synthesis of known or unidentified nutrients or in a lessened competition by the microorganisms for nutrients, the host benefiting from both.

Stimulating Effect of Products of Actinomycetes upon Plants and Bacteria

Koaze demonstrated that the culture filtrate of a streptomyces had a strong promoting effect on the germination of rice plants, in a dilution of 10^{-5} to 10^{-7}. A crystalline substance was obtained by ethyl acetate extraction and chromatography on aluminum oxide which was active in concentration of 0.1 mg/ml. It was a neutral substance, soluble in alcohol, benzene, chloroform, and water. On analysis it gave $C_{10}H_{16}N_2O_2$. It was found to be L-prolyl-L-valine anhydride (diketopiperazine). The substance had no antibiotic activity.

In a study of the effect of antibiotics upon plant growth Koaze *et al.* (1957) found that certain antibiotics showed a similar promoting effect; neomycin B and sarkomycin had a marked effect on the growth of broad beans.

Mention may also be made of the growth-promoting effect of certain actinomycetes upon cellulose-decomposing and nitrogen-fixing bacteria (Sanborn, 1926). The mechanism of this action is still insufficiently understood.

Ciferri and Machado noted, during the isolation of an antibiotic produced by a culture of a streptomyces belonging to the *S. griseus* group, a considerable yellow-green fluorescence of the metabolic liquids. The riboflavin potency of such liquids was assayed with mutant strains of *Lactobacillus casei* and *Leuconostoc mesenteroides*, giving an activity corresponding to a riboflavin content of 0.9 to 1.2 μg/ml. The fluorescent pigments could be easily extracted with *n*-butanol and purified by absorption on charcoal and elution with aqueous pyridine. Paper chromatography of such preparations and of the original broths showed at least four spots characterized by fluorescence under Wood's light but none could be identified either with riboflavin or its nucleotides or decomposition products. Only spot 2 reacted with ninhydrin. When the spots were cut off the chromatograms and tested individually, only one spot, characterized by a blue fluorescence, proved capable of sustaining the growth of *L. casei.*

Rubentschik *et al.* found that various streptomyces, such as *S. griseus, S. coelicolor,* and *S. globisporus,* when grown in cultures with other organisms, are capable of forming volatile materials which stimulate the growth of *E. coli, B. subtilis, B. mesentericus,* and other bacteria. Every *Streptomyces* species was said to exert a characteristic effect, *S. griseus* stimulated the growth of *Azotobacter.*

Grossbard reported that various antifungal substances produced by actinomycetes exert stimulative effects upon the growth of fungi, within a concentric circle adjoining the zone of inhibition. Pigmentation was intensified in cultures of *Verticilium dahliae, Helminthosporium victoriae,* and *Fusarium oxysporum.* The last usually fails to produce a pigment on synthetic media, but in response to the metabolites of certain streptomyces cultures, a pigment was formed in optimal media; a considerable intensification of the pigment (lycopersin) occurred. *Colletotrichum atramentarium* responded to the same streptomyces metabolites by an acceleration in the maturing and by a greater density of stromata. *Ceratostomella ulmi* and *Streptomyces scabies* responded by more rapid sporulation.

The favorable effect of certain streptomyces upon the sporulation of other cultures of the same genus has been demonstrated by Dondero and Scotti. These results led to the conclusion that actinomycete metabolites contain specific stimulatory substances in addition to inhibitory substances, or that the substances may be growth-inhibiting at one concentration and growth-stimulating at another.

The significance of these phenomena in natural processes in general and in the soil in particular is still to be elucidated. This is also true of the stimulating effect of actinomycin upon the growth of some strains of rhizobia and its inhibiting effect upon others, notably the slow growing strains (Trussell and Sarles).

CHAPTER 13

Production of Pigments

Among the most characteristic properties of actinomycetes is their ability to produce a great variety of pigments, both on organic and on synthetic media. This pigment-producing capacity is so characteristic as to serve for the naming of a large number of the species, notably those belonging to the genus *Streptomyces*. Very few of these pigments are pure. Most of them are mixed, with gradual transition from one color of the rainbow to another.

Conn and Conn emphasized the value of pigmentation in classifying actinomycetes. Stapp isolated from various soils 477 cultures of streptomyces. Their growth on glycerol-asparagin agar gave the following pigmentation: 31 per cent of the colonies were gray in appearance; 19 per cent yellow (cream to orange); 18 per cent brown; 17 per cent white; 8.4 per cent red; 4.2 per cent blue; 1.3 per cent green; and 0.8 per cent black.

von Plotho (1948) divided the actinomycetes into four large groups according to the pigments produced: 1. red-yellow; 2. red-blue; 3. red-brown; 4. colorless. Some of the pigments possess indicator properties: at an acid reaction, the red pigment predominates; at an alkaline reaction, the second pigment prevails. The colorless group have given the greatest number of antibiotic-producing strains. Members of the red-blue group produce strong pigments but weak antibiotics. Members of the red-yellow group produce both strong pigments and highly active antibiotics. The members of the red-brown group produce neither true pigments nor active antibiotics.

Only very few of the pigments of actinomycetes have been studied in detail. Some are soluble in water, others are soluble in alkalies, and still others in organic solvents, such as alcohol or chloroform (Table 48). These pigments have been divided into three groups: anthocyanins, carotenoids, and melanins. The red-blue pigments of the actinomycetes belong to the first group; red-orange-yellow pigments belong to the second; the black and brown pigments belong to the third. Some of the pigments are fluorescent, and some change in color with a change in reaction of medium. Some are intracellular; others are exocellular and dissolve readily in the medium. Some of the pigments are produced only on certain specific media, notably synthetic media; others are produced on a variety of media. In many cases, a change in the composition of medium results in a change in the nature of the pigment.

The pigments of actinomycetes are usually described in terms of various shades of blue, violet, red, rose, yellow, green, brown, and black. The shades of color are also frequently indicated, as light gray, deep gray, mouse gray. The pigments may be concentrated either in the vegetative mycelium or in the aerial mycelium and spores.

The pigment-producing property of actinomycetes is variable, depending upon the nature of the medium, the age of culture, and its previous cultivation. The insoluble pigments are more constant in nature than the soluble kinds. The formation of water-soluble brown to black pigments on organic media has been used to designate the chromogenic streptomyces. The tyrosinase action characteristic of these organisms was believed by Beijerinck to explain the mechanism of the production of this pigment.

Müller first studied the pigment produced by *S. coelicolor*. It is dark blue and diffuses readily into an alkaline medium. If the reaction of the culture changes to acid the pigment becomes red. This pigment was found to be produced on synthetic media with starch, sucrose, and other carbon sources. This is the reason for the designation of the culture variously as *S. violaceoruber* and *S. tricolor*. Beijerinck (1914) described a culture, *A. cyaneus*, now classified with the nocardias, which produced a pigment similar in its properties to the anthocyanins. This pigment was recently designated as litmocidin.

Lieske recognized two types of pigments among the actinomycetes: (a) the chromophores or pigments which are not excreted from the mycelium into the medium, and (b) the chromopars or pigments which are readily excreted. The first group comprises the pigments found in the vegetative mycelium grown on synthetic media; these are yellow, orange, red, blue, violet, brown, black, and green. The aerial mycelium of these cultures may be white, rose, lavender, red, yellow, orange, green, or gray. The soluble pigments are usually yellow, blue, and red; occasionally they are green, orange, or brown.

Kriss established that even the chromophore pigments are partly dissolved in the medium, possibly because of the lysis of the mycelium and the spores. Some of these pigments are insoluble in water and are bound to the proteins. Others are dissolved in the fats and lipoids of the cell. Some may be water-soluble, but are unable to pass through the living cell plasma; on the death and lysis of the cell, these pigments may dissolve into the medium. The solubility of the chromopar pigments in water is due to the greater penetration of the pigment through the cell wall.

TABLE 48

Solubility of actinomycete pigments (von Plc[illegible])

Group	Ether	Chloroform	CS_2	CCl_4	Butyl alcohol	Water
Red-yellow	+	+	+	+	+	−
Red-brown	−	−	−	−	+	+
Red-blue	−	−	−	−	−	−

Kriss suggested classification of the pigments of actinomycetes into four types:

I. Pigments soluble in water and in 96 per cent alcohol; these are capable of passing through the living cell plasma. This group has been subdivided into (a) anthocyanins, soluble only in water, and (b) hydroactinochromes, soluble in water and in alcohol.

II. Lipoactinochromes, insoluble in water but soluble in alcohol and in other organic solvents.

III. Pigments insoluble both in water and in organic solvents.

IV. A combination of water-soluble and water-insoluble pigments.

Lieske isolated a culture of a streptomyces that produced a carmine-red pigment that, when boiled in dilute acid, became soluble in alcohol and in ether. Certain actinomycetes produce a red pigment that is made soluble only by the action of concentrated HCl; on treatment with H_2SO_4 it is changed to a blue-green pigment. *N. polychromogenes* produces a red pigment, soluble in chloroform, ether, and acid, but not in alcohol, glycerol water, or dilute alkali; this pigment is also changed to blue-green by H_2SO_4.

Certain light yellow pigments produced by actinomycetes are insoluble in organic solvents, but are soluble in dilute KOH solution; they are changed, on treatment with concentrated H_2SO_4, first to green, then to dark brown. The yellow-red pigment of *N. corallina* was identified as belonging to the lipochrome group of fat-soluble pigments.

Nature of the Pigments

Anthocyanins

These pigments are readily soluble in water and in aqueous glycerol, alcohol, and other water-containing solvents. They are insoluble in absolute alcohol, chloroform, and other organic solvents. They are red in dilute acid solutions and blue in alkaline solutions. The neutral point is about pH 7.5.

The blue pigment of *S. coelicolor* has been extracted with cold and hot water as well as with alcohol. This pigment became red when treated with acid, and green when treated with 25 per cent alkali solution. Addition of lead acetate to the aqueous solution of the pigment brought about the formation of a violet precipitate. Müller (1908) first suggested the similarity of this pigment with anthocyanin. Kriss confirmed these results fully. Krassilnikov also confirmed the results and emphasized that the anthocyanins or allied pigments are characteristic of several actinomycetes. He pointed out that all the blue pigment-producing actinomycetes belong to *S. coelicolor* Müller. The cultures all produce a blue pigment which diffuses into the medium, conferring upon the medium the corresponding color. If the reaction of the medium changes to acid as a result of the growth of the organism, the color of the culture becomes red; on alkalinization of the medium, the color again turns blue.

According to Waksman, the pigment produced by *S. violaceoruber* behaves as an indicator, being red in an acid medium and blue in an alkaline medium; the change in pigmentation takes place at pH 6.6. According to Frampton and Taylor (1938), the pigment produced by *S. violaceoruber* is an anthocyanin; it was isolated as the crystalline picrate. These workers considered the pigment as a rhamnose glucoside, the carbohydrate groups being rhamnose and glucose. The sugars were believed to be separately attached to the anthocyanidin residue.

Jean Conn concluded that the two blue pigments produced by *S. coelicolor* and *S. violaceoruber* are not identical; the first is similar but not identical to azolitmin. On the basis of this differentiation, she believed that the two organisms represent distinct species. Oxford and Erikson *et al.*, however, could not accept the phenazine or anthocyanin nature of these pigments.

Tonolo *et al.* (1954) described the isolation, from a culture related to *S. coelicolor*, of a pigment designated as streptocyanin. The course of pigment production in a glycerol medium is shown in Figure 79. The pigment was soluble in acetone, pyridine, and dioxane. It colored blue-violet with H_2SO_4 and decomposed at 290 to 300C°. It gave a band in the visible spectrum. A quinoid structure was postulated for the pigment. It showed antibiotic activity.

Green Pigments

Actinomycetes were found to produce soluble green pigments, which is the reason for such species names as *S. viridis*, *S. viridochromogenes*, and *S. verne*. Some of these pigments are soluble in glycerol and in alkaline solution, but not in organic solvents. The color of the pigment in water is green; in glycerol, yellowish green. The composition of the medium influences the nature of this pigment. Green pigments produced by actinomycetes were also reported by other investigators.

One of the most interesting of the green pigments was recently studied by Chain and Tonolo. They isolated a culture of a streptomyces that produced on yeast agar an intense green nondiffusible pigment. This pigment was also formed in submerged,

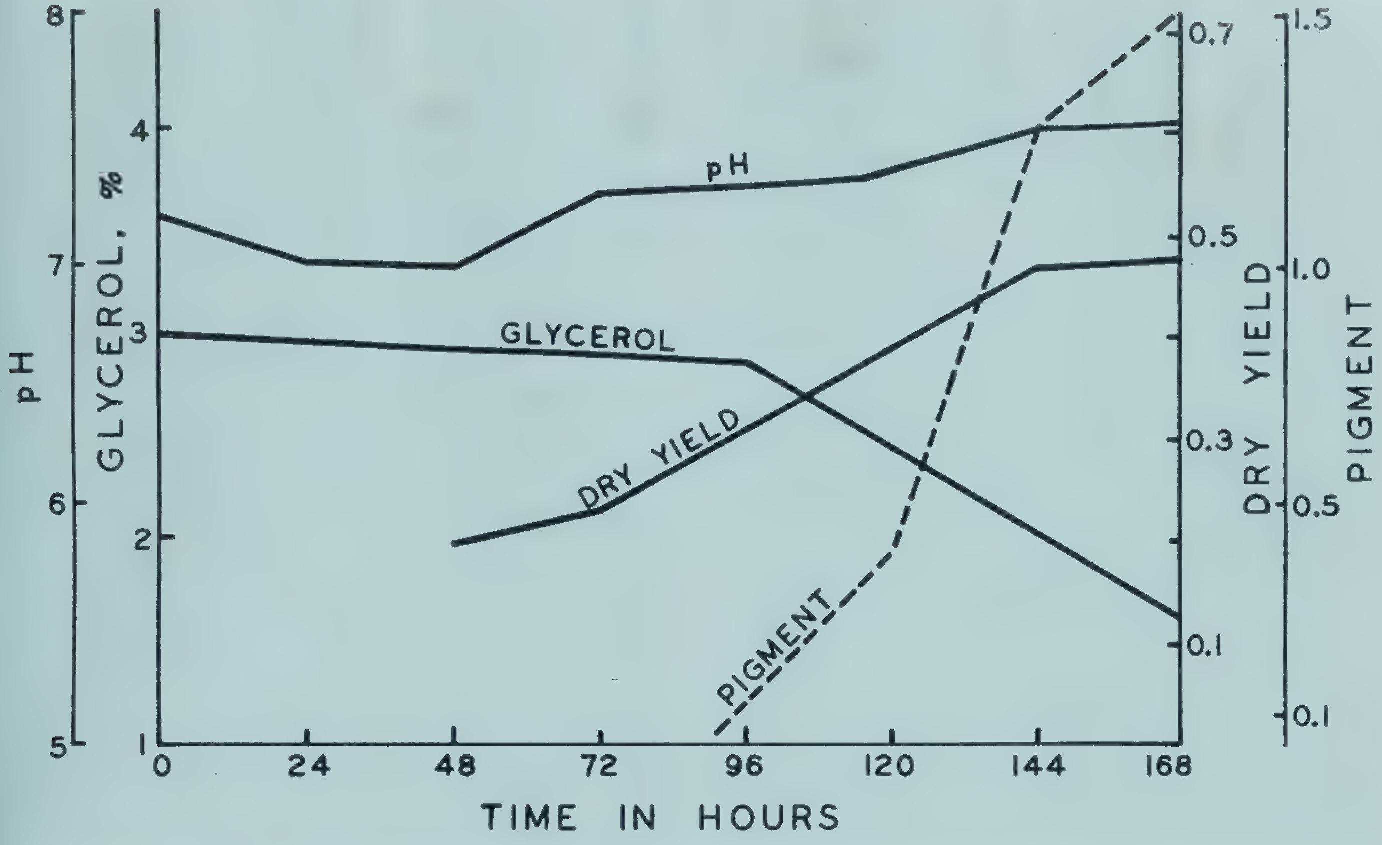


FIGURE 79. Course of fermentation by streptomyces in 200-liter tank (Reproduced from: Tonolo, A *et al.* Rend. ist. sup. sanita **17:** 958, 1954).

well-aerated cultures, in a glucose-containing medium, at pH 7.0. The pigment was extracted from the mycelium with ethanol, then transferred into butyl acetate, and the solution percolated through a column of alumina. The column was developed with 95 per cent alcohol, followed by absolute alcohol. Three bands were formed. The main, or middle, band was crystallized, yielding 100 to 200 mg of solid material per kilogram of moist actinomycete growth. This pigment was named *ferroverdin.* The formula assigned was $C_{30}H_{24}O_8N_2Fe$. The pigment was found to be insoluble in water and in benzene, chloroform, and certain other organic solvents. It was soluble in methanol, ethanol, acetone, and glacial acetic acid. It was reduced by hydrogen gas, in the presence of a proper catalyst, to a colorless substance. The iron was closely bound, but, after catalytic reduction, it appeared in ionic form.

Hydroactinochromes

This group, according to Kriss, comprises pigments that are soluble in water, 96 per cent alcohol, and chloroform, but not in ether, acetone, or CS_2. In a natural state, they are violet. In dilute acid solutions, they are orange; in an alkaline solution, dark violet or blue. These pigments are usually found in cultures admixed with other pigments, red or orange in color. Krassilnikov included among the cultures producing this type of pigment *S. violaceus* Gasperini and *S. violaceus niger* Waksman and Curtis. The first is said to produce only a small amount of the orange pigment, whereas the second also forms a dark melanin pigment.

According to Kriss and Krassilnikov, no pigment of this type was found in the nocardia cultures, although they reported that one such culture isolated by Berestnew had the capacity to produce such pigments.

Lipoactinochromes

These pigments are soluble in alcohol and in other fat solvents. They are red, orange, or yellow in a natural state. Kriss divided them into two subgroups: (a) Bright orange pigments soluble in petrol ether. The color does not change in an acid solution, but becomes lilac in an alkaline solution. The pigment is readily dissolved in CS_2. (b) Pigments insoluble in petroleum ether. They give a rose-red color in alcoholic solution. In dilute acid solutions, the color is red; and in alkaline, yellow.

This group of water-insoluble pigments includes the carotenoids. These are produced by the red, orange, and yellow species. Reader demonstrated two such pigments among actinomycetes, one of which was designated as corallin, an ether solution of which gave two bands of absorption in the spectrum.

Rhodomycin, another red pigment, was studied by Brockmann and Bauer (1950).

Prodigiosin Pigments

A prodigiosin-like pigment, yellow in an alkaline solution and red in an acid solution, was isolated by Dietzel from the mycelium of the organism producing actinorhodin. The dry mycelium was treated with methanol, then shaken with dilute alkali. The yellow pigment remained in the butanol solution, whereas the blue pigment dissolved in the alkali. The butanol was distilled off and the residue was dissolved in benzol and chromatographed on an Al_2O_3 column. The deeply red zone was treated with methanol. On shaking with $NaHCO_3$ solution, the pigment changed to yellow. The pigment proved to be prodigiosin-like in nature. The chemical formula, based on elementary analysis, was $C_{25}H_{36} \cdot O_5N_3Cl$. Several other streptomycetes produced similar pigments. It was suggested that the lipoactinochromes X and B of Kriss belong to this group of pigments.

Arcamone *et al.* (1957) confirmed Dietzel's results concerning the production of a prodigiosin-like pigment by certain streptomyces, notably *S. ruber* and *S. roseodiastaticus*. This pigment had antibiotic properties against gram-positive bacteria.

Brown-black Pigments

Various actinomycetes, notably species of *Micromonospora* and certain streptomycetes, produce a pigment that ranges from orange-brown to brown to black. Beijerinck assumed that the brown substance was a quinone. Waksman, Rubentschik, and others suggested that it is a result of the action of the enzyme

TABLE 49

Growth and soluble pigment production of S. griseus *in calcium salts of alpha-hydroxy and dibasic acids* (Benedict and Lindenfelder)

Calcium salt	Streptomycin-producing strains of *S. griseus*					
	Waksman 3496		Waksman 9		Carvajal 2060	
	Growth	Pigment	Growth	Pigment	Growth	Pigment
Citrate	+	Yellow	+	Yellow	++++	Light yellow
Malate	++++	Green	++++	Green	++	Light yellow
Lactate	+	Light yellow	+	Amber	+	Light yellow
Tartrate	−	—	−	—	−	—
Succinate	+++	Deep yellow	+++	Deep yellow	+	None
Malonate	+	Light yellow	±	None	+	None
Fumarate	±	None	±	None	±	None

tyrosinase. The majority of the "chromogenic" actinomycetes produce such pigments on organic media. Certain species produce these pigments also on synthetic media. Some of the brown to black pigments remain in the mycelium ("melanin"); others dissolve in the medium. The nature of the medium greatly influences the nature and intensity of the pigment produced.

Percival and Stewart made a detailed study of the mechanism of melanin formation. They found that in the presence of tyrosinase, the formation of melanin from tyrosine is apparently due to the oxidative formation of the red indole quinone through the action of the enzyme. The subsequent reactions, the formation of 3,4-hydroxyindole and its further oxidation to melanin, are able to take place merely in the presence of molecular oxygen, and without the intervention of any enzyme.

Environmental conditions, notably the degree of oxidation and the temperature of incubation, influence the formation of pigments. Aerobic conditions and lower temperatures (7–15°C) favor pigment formation in the culture. At 37°C pigment formation is greatly diminished.

Role of Pigments in the Life and Metabolism of Actinomycetes

Various hypotheses have been proposed to explain the role of pigments in the growth of the microbial cell. Their function in the respiratory mechanisms of the cell has been suggested. Some have claimed for them a role in the defense mechanism of the cell against the action of foreign cells or against the effect of sun rays. The recent interest in the subject of antibiotics has tended to concentrate attention upon these substances that are pigmented in nature.

Benedict and Lindenfelder (1951) have shown that the different varieties of *S. griseus* possess characteristic pigment-producing capacities on special media. Some of the streptomycin-producing strains are able to form yellow pigments on synthetic calcium malate media and green pigments on calcium succinate media. Grisein-producing strains are unable to produce such pigments (Table 49).

Antibiotic Pigments

A large number of actinomycetes produce pigmented antibiotics, which recently have received much consideration. Benedict proposed a system of classification of pigmented antibiotics produced by actinomycetes (Table 50). Some of these groups deserve more detailed consideration.

Actinomycins

Actinomycin was the first antibiotic isolated in a pure state from a culture of a streptomyces. It crystallizes from ethyl acetate or from acetone-ether mixtures as red platelets, m.p. 250°, $[\alpha]_{D}^{25}$ ($C = 0.25$ per cent in ethanol) $-320 \pm 5°$. It is soluble in chloroform, acetone, ethanol, hot ethyl acetate, carbon disulfide, and benzene, but only slightly soluble in water or ether. It is stable in aqueous alcohol when boiled for 30 minutes, but unstable in dilute acid or alkali. An alcoholic solution of actinomycin gives no coloration with ferric chloride; it shows characteristic light absorption in the visible region ($E_{1\,\mathrm{cm}}^{1\%} = 200$ at 450 μ) and in the ultraviolet region ($E_{1\,\mathrm{cm}}^{1\%} = 216$ at 215 mμ). It is highly active against certain gram-positive bacteria. Waksman and Tishler found that 10 μg given intraperitoneally or subcutaneously, killed 20-gm mice in 24 to 48 hours.

Various forms of actinomycin have since been isolated. Interest in this group of antibiotics has grown especially since it has been demonstrated that they exert a marked effect in the treatment of certain forms of cancer. The chemical studies of Dalgleish *et al.*, of Brockmann and Grubhofer, and of many others added greatly to our understanding

TABLE 50

Classification of pigmented antibiotics of actinomycetes (Benedict)

I. Yellow to greenish yellow to orange.	
1. Low solubility in water, insoluble in ether, soluble in other organic solvents.	
a. Golden yellow base, $C_{22}H_{23}N_2O_8Cl$, low toxicity, active against bacteria and larger viruses.	Chlortetracycline
b. Yellow amphoteric compound, $C_{22}H_{24}N_2O_9$, low toxicity, active against bacteria and viruses.	Oxytetracycline
c. Greenish yellow weakly basic, $C_{30}H_{34}N_4O_4$, strongly antifungal.	Fradicin
2. Insoluble in water, slightly soluble in ether, soluble in other organic solvents.	
a. Yellowish substance, active against yeasts and filamentous fungi.	Actinone
b. Golden yellow compound, $C_9H_{10}N_2O_2S_2$.	Aureothricin
c. Brilliant yellow neutral compound, $C_8H_8N_2O_2S_2$, active against various bacteria and fungi.	Thiolutin
d. Orange yellow, $C_{14}H_{12}N_4O_4S_4$.	Thioaurin
e. Yellowish orange basic substances, $C_{31}H_{36}N_4O_8 \cdot 3HCl$ (A), highly active and extremely toxic.	Xanthomycins A and B
f. Saffron-yellow weakly acidic, $C_{23}H_{18}O_6$, active against gram-positive bacteria.	Resistomycin
3. Soluble in water and in organic solvents other than ether.	
a. Yellowish orange, active against various bacteria.	Luteomycin
b. Yellowish green neutral, active against gram-positive bacteria.	Actinomyceline
c. Yellowish weakly acidic; active against yeasts and filamentous fungi.	Flavacid
4. Insoluble in water and ether, soluble in other organic solvents.	
a. Yellow acid substance, acid stable, active against gram-positive bacteria.	Griseolutein
b. Yellow substance; antifungal and trichomonadicidal.	Trichomycin
II. Yellowish red to dark red, blue and purple. With the exception of grisein and the microcins, all are primarily active against gram-positive bacteria.	
1. Practically insoluble in water, slightly soluble in ether, and soluble in other organic solvents.	
a. Red cyclic peptides, $C_{40-41}H_{56-57}N_{7-8}O_{11}$, very toxic	Actinomycins
b. Strongly resembling actinomycin, possibly identical.	Actinoflavins
2. Acid-base indicators, changing from red to blue	
a. Non-nitrogen-containing quinone, $C_{28}H_{20}O_{10}$, alkali-soluble, ether-insoluble.	Actinorhodin
b. Anthrocyanin-like pigment, soluble in water and ether.	Litmocidin
c. Low nitrogen-containing compound; red form, water-insoluble, ether-soluble.	Rhodomycetin
d. Amphoteric substituted quinones, low water solubility.	Rhodomycins A and B
e. Reddish purple pigment.	Coelicolorin
3. Red color due to a metallic element	
a. Weak acid, containing iron and amino acids, $C_{40}H_{61}N_{10}O_{20}SFe$, soluble in water and phenol.	Grisein

Table 50—*Continued*

b. Impure, grisein-like factor, production influenced markedly by iron, narrower spectrum than grisein.	Antibiotic 3510
c. Grisein-like substances.	Albomycin
4. Possibly containing pyrrole nuclei	
a. Red, weak base, $C_{65-67}H_{96-104}N_{18}O_{15}$. Sparingly soluble in water and ether.	Nocardianin
5. Violet pigment	
a. Soluble in ether, water, and alcohols	Rhodocidin
6. Partially purified compounds	
a. Neutral, reddish purple powder; antifungal and antibacterial.	Microcin A
b. Acidic, yellowish red powder; antifungal and antibacterial.	Microcin B

of the chemistry and activity of this group of antibiotics (Waksman, Katz and Vining, 1958).

Actinorhodin

Brockmann and Pini, and von Plotho isolated, in 1947, a culture of streptomyces which produced a polyoxyquinone pigment, designated as actinorhodin. It was soluble in acetone, pyridine, and dioxane. It gave a blue color with H_2SO_4, decomposing at 270°C. This pigment formed two distinct bands in the visible spectrum. It was active antibiotically. The antibiotic is believed to possess a quinone structure, having three free hydroxyl groups, one carboxyl group, and two hydroxyl groups adjacent to a carboxyl group (Brockmann and Hieronymus). Shockman and Waksman found that rhodomycetin is similar to actinorhodin. The antibiotic shows a blue color in alkaline solution, dark blue in sulfuric acid, and becomes red-violet on addition of boric acid.

Thiolutin, Aureothricin, and Thioaurin

This group of antibiotics is characterized by the presence of sulfur in the molecule.

Tanner *et al.* have shown that thiolutin ($C_8H_8N_2O_2S_2$) crystallizes as brilliant yellow needles which have no definite melting point but darken at about 255°C. It is soluble in alcohol, chloroform, glacial acetic acid, pyridine, and dimethylformamide; slightly soluble in ether, petroleum ether, and benzene; and sparingly soluble in water. It is very stable in acid solution and withstands heating for 1 hour at 100°C. It is active against a variety of gram-positive, gram-negative, and acid-fast organisms, as well as fungi, *Endamoeba histolytica*, and certain hemoflagellates.

Aureothricin also belongs to this group.

Bolhofer *et al.* obtained bright orange-yellow crystals of thioaurin ($C_{14}H_{12}N_4O_4S_4$) which melted with decomposition at 179 to 181°C. Thioaurin is relatively insoluble in water and in many organic solvents. It inhibits the growth of various bacteria, but unlike thiolutin, it shows little activity against fungi.

Coelicolorin

This is a purplish red powder, m.p. 142 to 146°C, which was found (Hatsuta) to be red at pH 5.0, purple at pH 6 to 7, and green at pH 8.0 or above. It is soluble in water at pH 8.0 or above. It is soluble in ethanol and other organic solvents, but insoluble in petroleum ether. It is active primarily against gram-positive bacteria.

Xanthomycins A and B

These yellow antibiotics were isolated by Thorne and Peterson. Rao and Peterson converted xanthomycin A to the crystalline hydrochloride, the elemental analyses giving $C_{31}H_{36}N_4O_8 \cdot 3HCl$. The antibiotics showed characteristic quinoid properties and contained four methoxyl groups and one methylimide group. Prolonged reaction shows the release of two primary amino groups. They were soluble in organic solvents. Acid hydrolysis gave ethanolamine, methylamine, and ammonia in a ratio of 2:1:1. Separation of A and B was accomplished by Craig countercurrent distribution. Xanthomycin strongly inhibits the growth of many gram-positive and gram-negative bacteria, but does not affect *M. tuberculosis* in dilutions as low as 1:2000. It is very toxic to experimental animals.

It is important to add further that, in the presence of certain metallic ions, some antibiotics may form pigmented compounds. The tetracyclines give green compounds with copper and nickel and red compounds with ferrous and ferric iron (Albert).

Luminescence of Actinomycetes

Certain actinomycetes have frequently been observed to give off luminescence under given conditions of culture. According to Rudaya (1958), there exists a correlation between the antibiotic activity of *S. rimosus* and its luminescence intensity in the ultraviolet. This is true especially when the cultures are grown on solid media. The rate of luminescence depends on the composition of the medium and on the age of the culture. The character of the luminescence was found to change with cultivation and storage, yellow luminescence being replaced by a blue one. Certain variants show only bright yellow luminescence. It has been suggested that luminescence analysis be used as a guide for the primary selection of *S. rimosus* strains by subjecting young cultures to ultraviolet irradiation on solid media which are favorable for antibiotic production.

CHAPTER 14

Antagonistic Properties

Phenomena of Association and Antagonism

Soils and water basins are inhabited by mixed microbiological populations. Among the members of these populations numerous associations and antagonisms occur. Complex populations also occur in other substrates, as in human and animal digestive tracts, where they may be responsible for certain mixed infections.

Pasteur, the biochemist, and DeBary, the botanist, were the first to emphasize the significance of possible antagonistic relations among different microorganisms living in such mixed populations. When two organisms were grown on the same substrate, one usually was found, sooner or later, to overwhelm and even bring about the death of the other. Such antagonistic activities embrace phenomena other than mere competition for or exhaustion of nutrients: they were found to be due largely to the formation of specific chemical substances (antibiotics) responsible for these effects.

The terms "antagonism" and "antibiosis" usually refer to the reduction in growth and activities of organisms living in association. The organisms thus affected may respond by exhibiting temporary or permanent modifications in their physiological characteristics; their morphology may be changed; a reduction in virulence may also result. Several types of antagonism are now recognized: 1. Antagonism *in vivo versus* antagonism *in vitro*. 2. Bacteriostatic or fungistatic, bactericidal or fungicidal, and lytic effects. 3. Antagonism of function *versus* antagonism of growth. 4. Direct and indirect antagonism. 5. One-sided or two-sided antagonism. 6. Isoantagonism and heteroantagonism, namely, antagonism between strains of the same species and antagonism among different species.

Among the various types of antagonism, the most definite and best understood are those that result in the formation of antibiotic substances, formerly spoken of as toxins, lysins, or bacteriolysins. The physical, chemical and biological properties of these substances vary greatly. Some are destroyed by boiling, others on exposure to light. Some are resistant to heat and to ultraviolet rays. Some are soluble in water, others in special solvents. Some are highly toxic to animals, others are relatively nontoxic.

Numerous early investigators observed the depressive effect of fungi upon bacteria and vice versa. It is sufficient to mention the observations of Tyndall (1876). However, the first well-recognized antibacterial preparation was that of *pyocyanase*, produced by *Ps. aeruginosa*, formerly known as *B. pyocyaneus*. Emmerich and Löw (1899) considered pyocyanase to be an enzyme system. Emmerich and Saida (1900) actually used it to destroy (dissolve) tubercle bacilli. Subsequent to these early studies, an extensive literature accumulated on the production of antibacterial substances by bacteria, culminating in the treatise by

Papacostas and Gaté (1928) on "Microbial associations and their therapeutic application" and the more recent systematic work of Dubos on tyrothricin.

The fungi as producers of antibacterial substances also received considerable attention (Waksman, 1945). The most spectacular work was that on *penicillin*, first recognized by Fleming (1929), and finally established by Chain, Florey, *et al.* (1940) as an important chemotherapeutic agent.

The actinomycetes were first recognized by Gasperini (1890) as potential destroyers of fungi and bacteria. Lieske reported, in 1921, that actinomycetes, especially the aerobic, spore-forming types (streptomyces), are not hindered in their growth by other organisms, but, on the contrary, are able, in spite of their slow development, to suppress the growth of almost all bacteria and fungi. This was found to be true on plates in which actinomycetes were seeded after the growth of fungi and bacteria had already occurred. When a culture of an actinomycete and a staphylococcus were mixed, streaked on agar plates, and incubated at 37°C for 24 hours, the plate became covered exclusively with the growth of staphylococci; on further incubation, however, the actinomycete assumed the upper hand and gradually replaced the bacteria. The growth of the actinomycetes, under these conditions, may have been better than in pure culture, thus giving the impression that the bacteria actually served as

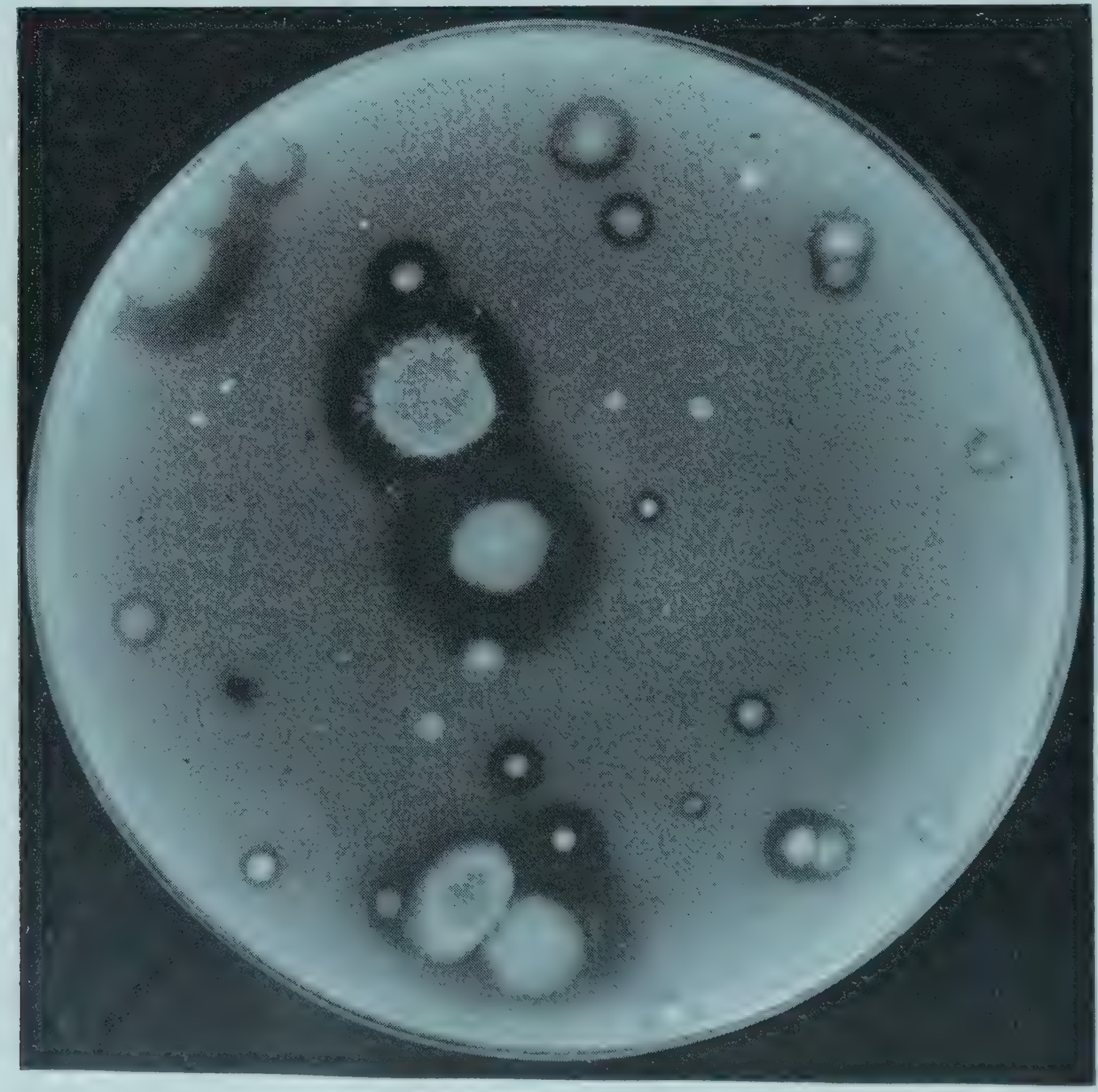


FIGURE 80. Production of clear zones on bacterial plates by antagonistic organisms

a growth stimulant. On the other hand, *Ps. pyocyaneus* repressed the growth of various actinomycetes in mixed culture, and even brought about destruction of the actinomycetes. The antagonistic effects of actinomycetes were ascribed by Lieske to their ability to excrete toxic substances that have the capacity to inhibit the growth of other organisms or even to destroy them.

Müller (1908) first observed that certain pigment-producing streptomyces (*S. coelicolor*) are able to suppress the development of yeast-like fungi. In a study of the associative and antagonistic effects of certain actinomycetes and fungi, Porter (1924) demonstrated that several organisms now known to belong to the genus *Streptomyces*, namely *S. tricolor*, *S. albus* var. *ochraceus* and *S. nigrificans*, produced marked inhibitory effects upon the growth of fungi. He suggested that such inhibitory action may aid in species identification.

Gratia (1924) definitely established the potentialities of actinomycetes as powerful antimicrobial agents. He and his associates isolated a preparation, designated "mycolysate," which was actually used in the treatment of many clinical cases caused by pathogenic bacteria, notably the typhoid organism.

Rosenthal isolated, in 1925, from dust, an actinomycete which he designated as the true biological antagonist of the diphtheria bacillus. He inoculated the surface of an agar plate with an emulsion of the bacillus and then introduced the actinomycete culture into several spots on the plate. After 2 days' incubation, the actinomycete colonies were surrounded by large transparent zones, whereas the rest of the plate was covered with the growth of the diphtheria organism. When an emulsion of this organism, previously killed by heat, was mixed with agar, and the mixture poured into the plates and inoculated with the actinomycete, the colonies of the latter were surrounded by clear zones. This demonstrated the fact that the actinomycete produced a lytic substance which diffused through the agar and dissolved the dead bacterial cells.

Forced Antagonism

The nature of the antimicrobial effects of different microbes is greatly influenced by the energy and nitrogen sources in the medium. Schiller believed that antagonism could be induced by using microbes as nutrients: in a dilute glucose solution without nitrogen, yeasts were said to be "forced" to kill and digest bacteria; if the yeasts were added to a fully developed bacterial culture, a bacteriolytic substance was produced which was also active outside of the yeast cells. However, when the bacteria were inoculated into cultures of yeasts suspended in distilled water, the yeasts were killed. Various efforts, however, to adapt cultures of actinomycetes by the process of "forced antagonism" to grow on specific bacteria failed to yield antibiotics that the organisms would not produce normally when grown upon proper artificial media.

The Soil as a Source of Antagonistic Actinomycetes

The soil may be considered as the major source of antagonistic organisms, especially actinomycetes. Extensive studies carried out in our laboratories on the enrichment of soil with *M. tuberculosis* did not lead to any special development of actinomycetes active upon these organisms. This can be explained by the fact that the production of antibiotics by an organism does not take place in response to certain specific nutrients. Thus, the activity differs from enzymatic processes that exhibit the phenomena of adaptation, whereby the organism benefits directly from a particular reaction. The activity is different, also, from that following soil enrichment which results in the development of nitrifying and nitrogen-fixing bacteria. The formation of antibiotics does not appear to be correlated, therefore, with the stimulation of reactions influencing the breakdown of nutrients or certain other oxidation and fixation processes.

Greig-Smith, in his studies on the occurrence of toxic substances in soil, gave a clear description of the antagonistic effect of actinomycetes against bacteria and fungi; the fact that actinomycetes grow only slowly in normal soils suggested the possibility that they comprise an important factor limiting bacterial development. It may be of interest to quote from his paper:

> "In making counts of soil-bacteria, it is not uncommon to find colonies of *Bac. mycoides* or of races of *Bac. vulgatus* spreading over the surface of the nutritive agar. Very often it will be noted that, while the majority of the colonies are covered by the spreading growths, there are a few that are untouched. The mycoides-colony may approach to within two, five, or ten millimetres, and then spread out and surround the colony, leaving a ring of clear agar medium. It is evident that there is some product secreted by these colonies which is obnoxious to the spreading colony, whether it be *Bac. mycoides*, *Bac. vulgatus*, or to spreading moulds such as *Penicillium* or *Aspergillus*. An examination of the colonies producing this toxic effect showed that the majority consisted of Actinomyces or Streptothrix as they have been called. Some of these darkened the medium and were apparently *Act. chromogenus*. Certain of these colonies were selected, and spotted upon fresh plates, in the centre of which bacteria with spreading colonies were planted. The white forms were found to be very toxic, while the dark forms were feebly toxic to the spreading *Bac. vulgatus*."

Evidence concerning the antagonistic potentialities of actinomycetes began to accumulate also as a result of a different approach. Millard and Taylor succeeded in controlling potato scab, caused by *S. scabies*, by the use of green manures and grass cuttings. When potatoes were grown in sterilized soil infected with *S. scabies*, scab was reduced by the simultaneous introduction into the soil of *S. praecox*, a saprophytic organism. By increasing the proportion of the saprophyte

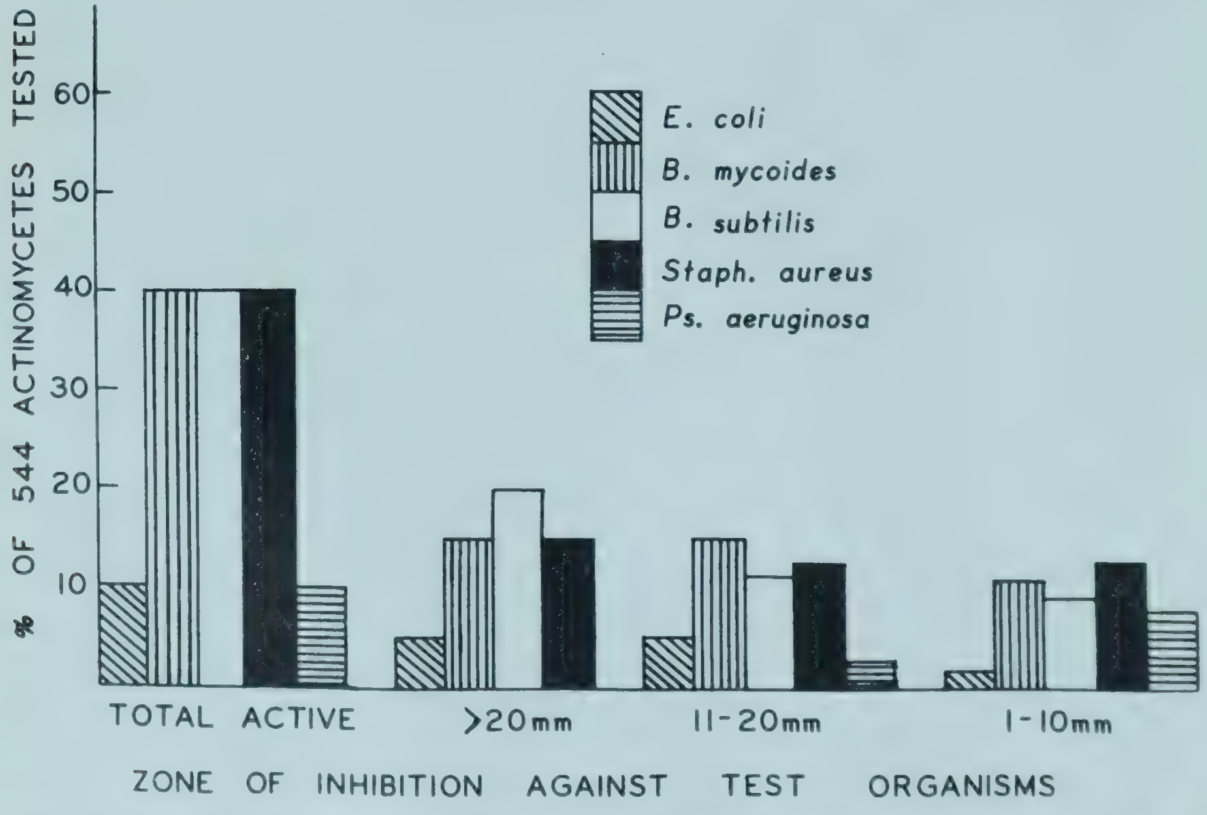


FIGURE 81. Distribution of antagonistic properties among freshly isolated actinomycetes. (Reproduced from: Rouatt, J. W., Lechevalier, M., and Waksman, S. A. Antib. Chemoth. 1: 189. 1951)

to the pathogen, the degree of scabbing on the test potatoes was reduced from 100 per cent to nil.

Goss found, however, that the general soil microflora rather than certain specific organisms had a controlling effect upon the development of scab; inoculation of soil with *S. praecox* alone gave negative results. Sanford and Cormack also were unable to obtain control of potato scab by the simultaneous inoculation with *S. scabies* and *S. praecox* of steam-sterilized soils or of natural soil enriched with green plant materials; these organisms were found to be perfectly compatible on potato-glucose agar as well as in a steam-sterilized soil. It was suggested that the control of scab on potatoes obtained by Millard was possibly due not to the direct action of *S. praecox* but to certain other undetermined microorganisms favored by the presence of the green manure or by other undetermined conditions.

In their studies on the decomposition of plant residues by pure and mixed cultures of microorganisms, Waksman and Hutchings showed that actinomycetes exert an antagonistic effect upon the activities of other microorganisms. Thus the ground work was laid for the systematic study of the antagonistic properties of actinomycetes and their ability to produce antibiotic substances.

Screening Programs

In search for microbes that have the capacity to produce antibiotics, two procedures have been generally followed. One is based upon the observation of bacterial plates that had become infected with a culture from the outside, usually dust, whereby the growth of the contaminant was surrounded by clear zones in which bacterial growth was inhibited. This was true of numerous observations made in microbiological laboratories, the most famous of which is that of Fleming on production of penicillin by a mold. The second method consists in isolating various organisms from natural substrates and testing them, by the agar streak method, for their ability to inhibit the growth of other bacteria and fungi. These

TABLE 51

Isolation of antagonistic actinomycetes from various substrates
(Waksman, Horning, Welsch, and Woodruff)

The organisms in group I were the most active antagonists, those in groups II and III had more limited antagonistic properties, and those in group IV showed no antibacterial effects with the methods used.

Source of organisms	Number of cultures isolated	Group I		Group II		Group III		Group IV	
		Cultures	Percentage	Cultures	Percentage	Cultures	Percentage	Cultures	Percentage
Fertile soil	74	20	27.0	5	6.8	1	1.3	48	64.9
Infertile soil	75	11	14.7	8	10.7	4	5.2	52	69.3
Potted soil	13	1	7.7	1	7.7	0	0	11	84.6
Soil enriched with *E. coli*	21	1	4.8	4	19.0	4	19.0	12	57.2
Soil enriched with bacteria	15	12	80.0	2	13.3	0	0	1	6.7
Lake mud	9	3	33.3	4	44.4	0	0	2	22.2
Compost	37	1	2.7	20	54.0	4	10.8	12	32.4
Total	244	49	20.1	44	18.0	13	5.3	138	56.6

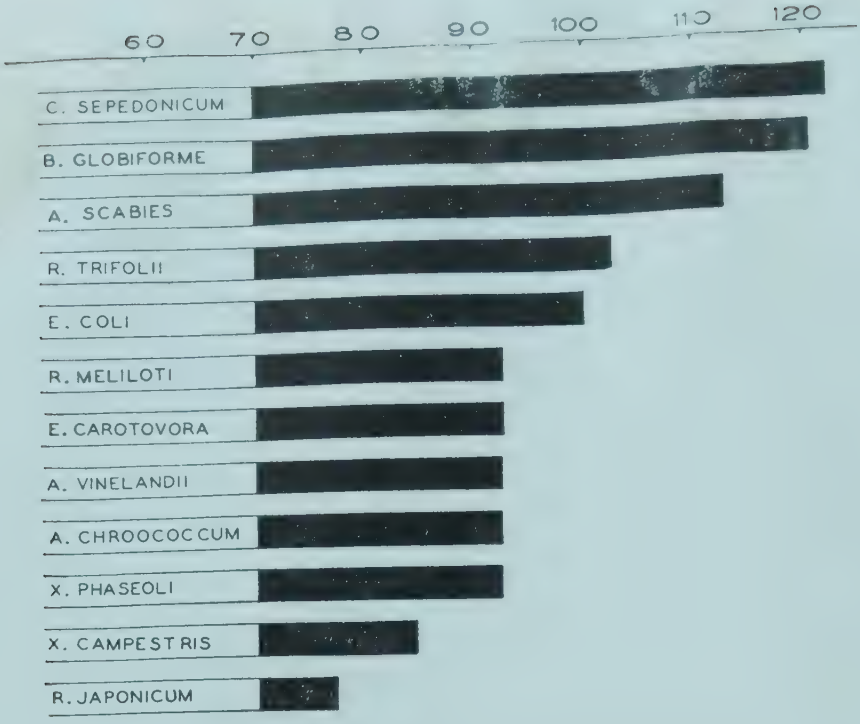


FIGURE 82. Relative susceptibility of test organisms to antagonistic actinomycetes (*E. coli* = 100) (Reproduced from: Landerkin, G. B. and Lochhead, A. G. Can. J. Res. **26C**: 505, 1948).

procedures, with numerous modifications, have found extensive applications in the study of the antagonistic properties of actinomycetes and have come to be known as screening methods.

In 1935, the first comprehensive survey for the occurrence of antagonistic forms among actinomycetes, largely soil inhabitants, was begun in Russia. Similar surveys of the occurrence of antibiotic-producing microorganisms, especially actinomycetes, were started in the United States in 1939. These surveys influenced greatly the whole subsequent history of antibiotics.

General Surveys

The first survey of the occurrence in soil of antagonistic actinomycetes was made by Nakhimovskaia. Out of 80 cultures of actinomycetes isolated and tested, 47 were able to exert antagonistic effects, but only 27 produced active substances. Gram-positive bacteria were inhibited, but not gram-negative bacteria or fungi. No relation was observed between antagonism and formation of pigments, manner of sporulation, or shape of spores. Since the capacity to produce antibacterial substances was possessed by only certain cultures, it was suggested that this property could be utilized for the systematization of species of actinomycetes. It was also suggested that various bacteria could be differentiated on the basis of their sensitivity to the actinomycetes.

Krassilnikov and Koreniako found that many species of actinomycetes, members of the group now considered as the genus *Streptomyces*, but not of *Nocardia*, produce substances that are strongly bactericidal against nocardias, mycobacteria, micrococci, and spore-forming bacteria, but not against gram-negative bacteria. Under the influence of these substances, the microbial cells are either entirely lysed or are killed without subsequent lysis. This chemical nature of the active substance was believed to be similar to that of lysozyme.

Kriss isolated a chemical agent from cultures of actinomycetes. The substance was found to be insoluble in ether, petroleum ether, benzol, and chloroform, and was resistant to the effect of light, air, and high

TABLE 52

Surveys of antagonistic actinomycetes isolated from soils and other natural substrata (Benedict)

Investigators	No. of cultures tested	Per cent of total active against			
		Gram-positive bacteria	Gram-negative bacteria	Acid-fast bacteria	Filamentous fungi
Nakhimovskaia	80	59	—	—	—
Waksman *et al.*	244	43	—	—	—
Welsch	164	46	—	—	—
Meredith	7642	—	—	—	1
Burkholder	7369	25	3	—	7
Landerkin *et al.*	660	48	8	20	21
Emerson *et al.*	772	52	—	—	47
Rouatt *et al.*	544	39	10	—	—
Cercos and Rodriguez	54	90	20	60	20
Kuroya *et al.*	1223	13	—	—	—

TABLE 53

*Distribution of antagonistic properties among actinomycetes** (Johnstone)

Zone of inhibition, mm	Per cent of cultures active against			
	B. subtilis	*E. coli*	*M. avium*	*M. phlei*
Nutrient agar				
20–35	21	6	6	23
10–19	46	3	35	35
1–9	3	13	29	16
0	30	78	30	26
Glucose asparagine agar				
20–35	15	0	0	6
10–19	28	6	35	70
1–9	35	14	8	10
0	22	80	57	14

* Cross-streak method used.

TABLE 54

Distribution of antibiotic activity of actinomycetes according to genus (Emerson *et al.*)

	Genus		
	Streptomyces	*Micromonospora*	*Nocardia*
Tested against bacteria:			
Number active	399	1	
Number inactive	370	2	
Number tested	769	3	
Tested against fungal pathogens:			
Number active	353	0	
Number inactive	399	6	8
Number tested	752	6	8
Tested against both bacteria and fungi:			
Number active against-			
bacteria only	149		
fungi only	109		
both	233		
neither	237		
number tested	728		

temperatures. Kriss also believed that the agent was similar to egg-white lysozyme.

Waksman *et al.* made a detailed survey of actinomycetes possessing antagonistic properties (Waksman, 1937, 1941, 1945). This led directly to the isolation of the first antibiotic produced by a member of this group of organisms, namely, actinomycin. Antibiotic-producing actinomycetes were found to be widely distributed in nature, especially in soils and in composts. Two hundred and forty-four cultures were isolated and tested. Of these, 106 or 43.4 per cent, possessed some antagonistic properties, and 49, or 20 per cent, were highly antagonistic (Table 51). An examination of a large series of well-identified cultures of actinomycetes kept for a number of years in a type culture collection showed similar relations (Welsch). The antagonistic forms were found to belong largely in the genus *Streptomyces.*

Burkholder isolated 7,369 cultures of actinomycetes from soil. Of these, 1,869 inhibited growth of *Staph. aureus,* 261 inhibited *E. coli,* and 514 showed an antagonistic effect against *C. albicans.*

With the growing interest in antibiotics throughout the world, numerous other surveys have since been made, as summarized by Benedict (Table 52). Most of these revealed that 20 to 50 per cent of all the cultures of actinomycetes tested possessed antagonistic properties. In some surveys the percentage was higher, in some lower. Stapp, for example, reported that 233 out of 477 cultures of streptomyces isolated from soil were active against *B. fusiformis*. The nature of the medium used for testing purposes is of great importance, as shown by Johnstone (Table 53). Most of the cultures were active against gram-positive, including acid-fast, bacteria; fewer were active against gram-negative bacteria and fungi. The majority of antibiotic-producing actinomycetes are found among the streptomycetes (Table 54).

Landerkin and Lochhead isolated from different soils 50 actinomycetes antagonistic to *E. coli*. When tested against different bacteria, those actinomycetes that possessed "the most intense antibiotic activity" were also active upon "the greatest number of bacterial species," in other words, had the widest antibiotic spectrum (Fig. 82). A detailed analysis of the results obtained by Landerkin *et al.* is presented in Table 55. Here as well, fewer organisms were active upon fungi than upon bacteria; of the latter, the gram-positive forms were most susceptible, the gram-negative least. There were also marked differences in the degree of sensitivity within each group; many more organisms were active upon *E. coli* than upon *Ps. aeruginosa*.

Of 1,117 cultures of actinomycetes tested by Aleshina and Makanovskaia, 44 per cent were active against staphylococci and 19.4 per cent against the plague organism. Of 170 cultures isolated and tested by Mukherje and Nandi, 40 per cent were active against gram-positive bacteria, 21.1 per cent against gram-negative bacteria, and 31.7 per cent against fungi. Different isolates belonging to the same species varied in their antagonistic properties. Some of the cultures that exhibited antagonistic effects when tested by the agar streak method, did not form, in liquid media, substances with the same kind of activity.

In a study of 70 strains of actinomycetes for their antagonistic effect upon root-nodule bacteria, Fogle and Allen found that 25

TABLE 55

Antibiotic activity of 600 actinomycetes isolated from northern Canadian soils (Landerkin, Smith, and Lochhead)

Number of cultures active against	Degree of activity*					
	+++	++	+	Inactive	Total active	Per cent active
Staph. aureus	140	84	87	349	311	47.1
E. coli	5	13	36	606	54	8.2
Ps. aeruginosa	0	0	3	657	3	0.5
M. tuberculosis	8	28	93	531	129	19.5
H. sativum	35	43	39	543	117	17.7
F. lini	2	14	46	598	62	9.4

* +++ = diameter of zone, 30 mm or more; ++ = 20–29 mm; + = 10–19 mm; inactive = less than 10 mm.

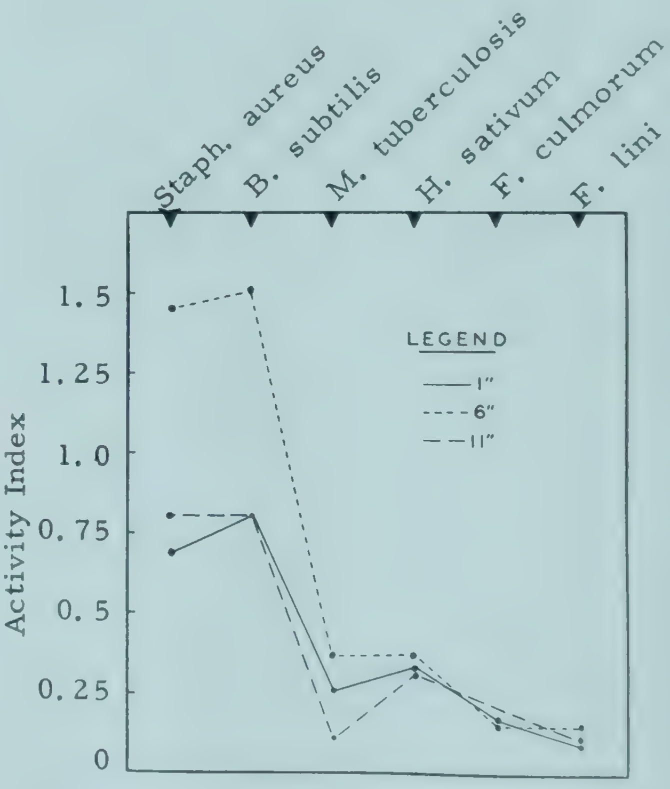


FIGURE 83. "Antibiotic index" of actinomycetes in relation to depth of soil (Reproduced from: Landerkin, G. B. *et al.* Can. J. Res. **28C**, 696, 1950).

strains were antagonistic to *Rh. lupini*. Other species of *Rhizobium* were inhibited by fewer strains: *Rh. trifolii* was inhibited by only 9 strains, and the cowpea rhizobia by only 1 strain.

In 1952, Poppe and Strutz isolated, from soil and other matter, 220 cultures of actinomycetes. Of these, 62 possessed antibacterial properties. Ten of the active cultures were found to exert a strong effect upon various gram-positive bacteria. Glycerol-glycocoll solution proved to be especially useful in the production of the antibiotic substances.

Lindner and Wallhäusser (1955) isolated 2,500 antagonistically active cultures of streptomyces from 40,000 soil samples. Their distribution in different soils is shown in Table 56. Of these, 77 per cent were active upon gram-positive bacteria (*Staph. aureus*), 40 per cent upon gram-negative bacteria (*E. coli*), 32 per cent upon mycobacteria (*M. tuberculosis* 607), and 18 per cent upon fungi (*A. niger*). Wallhäusser (1951) proposed a series of charts for representing the mode of growth inhibition of one organism by another.

The results obtained in these surveys were found to depend upon the test organisms used, the composition of the media upon which the organisms were grown, and various experimental conditions. Most of the active cultures belonged to the genus *Streptomyces*. However, Endo, who tested the activity of 116 strains of *Nocardia* upon 10 bacteria and 6 fungi and yeasts, found that 27 per cent of all strains were active upon at least one of the test organisms. The conclusion was reached that the *Nocardia* group has as high percentage of active strains as *Streptomyces*. The genus *Micromonospora* as well was found to be capable of exerting antagonistic effects against certain bacteria.

Craveri *et al.* (1957) isolated 500 cultures of streptomyces from various soils in Italy. Just about a half had the capacity to inhibit microbial growth on solid media. Among the latter, about one-third produced an antibiotic substance when grown in liquid media under submerged conditions. The percentage of the cultures active upon gram-positive bacteria only was higher than that of strains active upon both gram-positive and gram-negative bacteria. No culture was found to be active only upon gram-negative bacteria. Of the cultures active only upon gram-positive bacteria, about 20 per cent were active upon *B. subtilis*, 20 per cent upon *Staph. aureus* only, and about 60 per cent upon both.

TABLE 56

Distribution of antagonistic streptomyces

(Lindner and Wallhäusser)

Nature of soil and vegetation	Per cent of active strains
Composts	8.6
Garden soils	7.4
Field soils	14.8
Pastures	18.6
Forest soils	17.2
Brown soil	7.4
River muds	3.8
Virgin soils	22.2

Jarikova *et al.* (1958) isolated 1,879 actinomycete cultures from soils of different regions of the Soviet Union; of these, 1,262 cultures, or 67.2 per cent, proved active under the conditions of their experiments. The largest number were found in the greenhouse soil of the Botanical Garden, in meadow-granular soils, and in steppe soils, as well as in light chestnut soils. The greatest number of cultures having activity were found in soils of eastern and southern regions.

Attention must be directed to the fact that generalizations concerning the characterization of certain types of soil by the occurrence of specific antagonists are hardly justified unless they are based upon detailed and oft-repeated investigations.

Antifungal Surveys

The ability of various actinomycetes to antagonize the growth of fungi has long

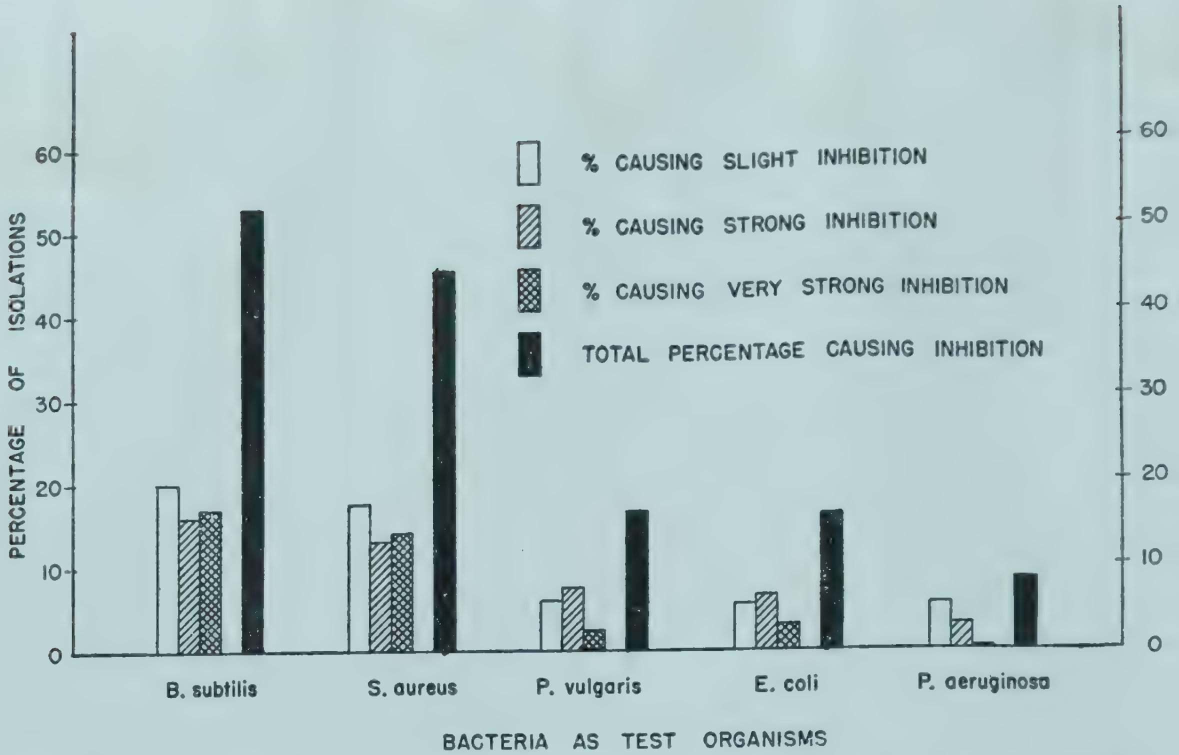


FIGURE 84. Degree of antibiotic activity with bacteria as test organisms (Reproduced from: Emerson R. L. *et al*. J. Bacteriol. **52:** 362, 1946).

been known. Major attention was directed toward their activity upon plant pathogenic fungi (Tius, 1932; McCormack, 1935). Alexopoulos was the first to study, in 1941, the distribution of antagonistic activities among actinomycetes against fungi. Of 80 cultures tested against *Colletotrichum gloeosporioides*, 17.5 per cent were strong inhibitors, 38.8 per cent were weak inhibitors, and 43.7 per cent had no effect at all. Meredith made a survey of the distribution of organisms antagonistic to *Fusarium oxysporum cubense* in Jamaica soils. The antagonists were not evenly distributed, 10 of the 66 soil samples giving 44 per cent of the antagonistic organisms. Those actinomycetes that were antagonistic to *Fusarium* when grown in their own soil-solution agar were not always antagonistic when tested in soil-solution agar prepared from another soil. A culture of an actinomycete isolated from a compost produced lysis of the *Fusarium*. When spores of both organisms were mixed in an agar medium, the fungus developed normally for 2 days but began to undergo lysis on the fifth day, large sections of the mycelium disappearing. On the seventh day only chlamydospores were observed. In 9 days the fungus completely disappeared, whereas the actinomycete was making a normal growth.

Leben and Keitt isolated a streptomyces that was antagonistic to various phytopathogenic fungi, but not to most bacteria. The active material was heat-labile, soluble in various organic solvents, and in water at pH 9.3. It inhibited the growth of fungi and of only very few bacteria. Of 3,788 actinomycete cultures isolated from soil by Cooper and Chilton and tested against *Pythium*, 896, or 23.6 per cent, showed some antagonistic effect upon the fungus. Certain actinomycetes were found to be responsible for the destruction, in soil, especially in partly sterilized soils, of the mycelium of *Ophiobolus graminis*, the cause of the take-all disease of wheat. The parasitizing and antibiotic effects of actinomycetes and other soil organisms were believed to be responsible for the check in the development of the *Ophiobolus* in natural soils.

Stessel studied the distribution in soil of actinomycetes active against pathogenic fungi. Dilutions of soil samples were added to suitable agar media, so as to permit 30 to 50 colonies to develop on each plate. The plates were incubated for 4 days at 26°C. Suspensions of spores of four mutually non-antagonistic phytopathogenic fungi were then sprayed on the plates. After 2 days' further incubation, 170 cultures of actinomycetes producing marked inhibition zones against all the test fungi were isolated. Plate cultures of the isolates were sprayed again but with separate suspensions of eight fungi and two bacteria. Twenty-one cultures, mostly actinomycetes, were selected on the basis of comparative inhibition. These organisms were grown in five liquid media in shaken flasks. The culture filtrates were tested for activity against *Glomerella cingulata*, by the use of a modified cylinder plate method. Sixteen cultures produced an antibiotic in one or more media. Of the 170 organisms originally isolated, five appeared to produce desirable substances, as determined by the above screening tests. Further studies on the antagonistic effects of actinomycetes upon plant pathogenic fungi have been made by Mukherjee and Nandi (1955) and others. Some actinomycetes are particularly active against yeasts (Takahashi, 1952). The activities of soil actinomycetes upon fungi pathogenic to man were examined by Schatz and Hazen.

Pridham *et al.* (1956) made an extensive survey of actinomycetes, largely streptomyces strains, for their growth-inhibiting effect against phytopathogenic bacteria and fungi. About 500 pure cultures were subjected to primary screening by three methods against a minimum of 12 test organisms. Based on their antimicrobial spectra, some 200 strains were selected as warranting further study and were grown in a variety of media in shake-flasks. The culture filtrates were tested for antibiotic activity by paper-disc assay against nine organisms. Fifty-two of the 200 strains showed promise. Culture filtrates from eight strains as well as two substances isolated from the mycelium of different strains were then tested in the greenhouse against certain plant diseases. Nine of the 10 preparations showed broad antifungal spectra in laboratory tests and activity against at least one disease in greenhouse tests. Phytotoxic effects were observed with some of the test materials. The details of the test are brought out in Table 57.

Correlation of Antimicrobial Activity and Pigmentation

von Plotho correlated pigment production by actinomycetes with their antagonistic properties. Two hundred and ninety-one cultures grown in colorless media were classified into four groups on the basis of pigments produced either in the mycelium or in the medium. Activity was determined by testing against *Mycobacterium eos*. Of the 61 cultures (21 per cent) showing activity, 21 were in the colorless group, 20 were in the red-yellow, 12 in the red-blue, and 8 in the red-brown group. By using media in which pigments could be seen readily, investigators might well try such a correlation to learn whether particular groups could be eliminated without further testing. A detailed analysis of the antibiotic pigments of actinomycetes was presented in Chapter 13.

Screening for Specific Antibiotic-producing Organisms

By using the technique reported by Waksman, Reilly, and Johnstone (1946) of adding a particular antibiotic to the medium before plating out soil samples, Umezawa *et al.* (1949) were able to demonstrate the presence

Table 57

Inhibitory activity of streptomyces against selected phytopathogenic bacteria and fungi (Pridham *et al.*, 1956)

Test organism	No. of streptomyces tested	No. of strains that produced inhibition zones with indicated diameter				
		0 mm	0–10 mm	10–20 mm	20–30 mm	30–50 mm
Agrobacterium tumefaciens	482	389	65	24	4	0
Bacterium stewartii	479	337	81	28	20	13
Corynebacterium fascians	479	170	106	121	52	30
Erwinia carotovora	381	337	32	11	1	0
Pseudomonas phaseolicola	477	327	59	56	21	14
Xanthomonas phaseoli	680	490	90	72	17	11
Ceratostomella ulmi	320	284	14	20	2	0
Cladosporium herbarum	319	257	31	27	4	0
Gibberella fujikuroi	66	21	26	18	1	0
Helminthosporium sp.	23	1	7	14	1	0
Mucor rammannianus	444	295	24	64	38	23
Trichoderma viride	320	300	17	1	2	0
Ustilago zeae	318	283	12	14	9	0
Fusarium oxysporum f. lycopersici + *Cl. herbarum*	246	88	1	93	41	23
Helminthosporium sp. + *G. fujikuroi*	66	27	22	15	2	0
Helminthosporium sp. + *T. viride*	297	162	6	70	29	30
T. viride + *G. fujikuroi*	164	84	14	27	29	10
Ustilago zeae + *Ceratostomella ulmi*	158	25	0	23	49	61

of a rather large number of specific antibiotic-producing strains. They found such strains to be present in four of the five soils tested. This would seem to indicate a wide distribution of such organisms.

In the process of screening thousands of soil samples from widely scattered geographical areas, Routien and Finlay found organisms producing certain antibiotics to be extremely common. Actinomycetes elaborating streptomycin, streptothricin, chloramphenicol, actinomycin, and xanthomycin-like antibiotics apparently have a world-wide distribution. The tetracycline-producing cultures have been isolated only a few times. One antibiotic was observed from only one culture isolated from one particular soil. These investigators found that certain antibiotics are produced by cultures of actinomycetes common in soils from somewhat localized areas. On the other hand, soils collected within a restricted area were found to yield a number of different antibiotics.

Routien and Finlay emphasized that variations in the degree of activity of different cultures are frequent. Some cultures may decline in potency. Some cultures may produce only small quantities of an antibiotic, which, on concentration, may be found to possess new and useful properties.

The wide distribution of antagonistic properties among actinomycetes has thus been definitely established. Members of the genus *Streptomyces* are most active. They occur abundantly in soil (Rouatt *et al.*, 1951). Even the plant pathogenic *S. scabies* possesses antagonistic properties (Ark and Oswald), although this organism, as well, is subject to the antagonistic action of certain fungi (Daines, 1937). Various nocardias are capable of exerting an antagonistic action, as shown by Uesaka and by others.

In summarizing the results of evaluation of antibiotic activities of 10,000 streptomyces cultures isolated from various substrates, Woodruff and McDaniel stated that, on the average, 25 per cent of the isolates were found to produce an antibiotic. Approximately 90 per cent of all the antibiotic-producing cultures formed streptothricin or closely related compounds. A half of the remainder produced streptomycin, one-third (of the other half) formed tetracyclines. Finally, most of those that remained could be identified with one of the various antibiotics which have been isolated from cultures of streptomycetes. In other words, 2,250 cultures gave streptothricin-like antibiotics, about 125 streptomycin, 40 yielded tetracyclines, 55 produced other previously described antibiotics, and only 30 yielded new antibiotics.

Test Organisms

A wide selection of organisms is commonly used in testing cultures for antibiotic activity. Frequently, a highly sensitive organism like *B. subtilis* or *Staph. aureus* is used. In a search for a specific antibiotic against a particular parasite, such as *M. tuberculosis*, the latter itself or a form closely related to it is used as the test organism. In most cases, however, various organisms are used, including gram-positive and gram-negative bacteria, saprophytic and parasitic fungi. Plate method techniques have been developed by Waksman and Reilly (1945), Waksman *et al.* (1947), and Williston *et al.* (1947).

Recently, actinomycetes possessing activity against viruses and experimental cancers have been investigated. For this purpose, special procedures are used, such as those involving bacteriophages (Jones and Schatz; Schatz and Plager, 1948). Out of 527 cultures of actinomycetes screened by Landerkin *et al.* (1957) for antiphage activity, eight inhibited the development of bacteriophages; of these eight, only one had an effect upon phages of two species of bacteria. None of the cultures tested had any effect upon the Newcastle virus.

Krassilnikov and Kofanova reported that nearly all actinomycetes possess antiphage properties. Some inhibit many phages and others are effective only upon specific phages. Actinomycetes show a characteristic antiphage spectrum. There is no relation between antivirus and antiphage antibiotics; therefore, the latter cannot be used as models for study of antivirus agents.

An interesting *in vitro* technique utilizing a modified agar plate diffusion method for the detection of antitumor activity was described by Miyamura. Ehrlich's ascites tumor cells are incorporated into a basal medium. The materials to be tested are placed in cups and allowed to diffuse through the medium; the cups are then removed and the plate is flooded with methylene blue; the agar is covered with a glass plate and incubated; the diameters of the blue zones are measured. Positive results were obtained with various anticancer agents including the antibiotics trichomycin and sarkomycin and 8-azaguanine. Other antibiotics of actinomycetes (tetracyclines, streptomycin, and neomycin) gave negative results.

Arai and Suzuki published a modification of the above method. Serial dilutions of the materials to be tested are placed in tubes. The tumor cells are added, the suspension is mixed thoroughly, and then buffered glucose agar containing methylene blue is added. The results are read after 3 hours' incubation. All antitumor substances tested, except 8-azaguanine, gave positive results. These substances included sarkomycin, carzinophilin, the gancidin complex, and others.

Rombouts (1953), Stevenson (1954), and Links *et al.* (1957) isolated certain cultures of streptomyces from soil that produced an antibiotic possessing the capacity of causing the swelling of hyphae of several fungi. The substance responsible for this swelling was designated as the "bulging factor." This substance, isolated from the medium, proved to be a streptothricin-like base readily soluble in water, but not in organic solvents. It was stable in an acid reaction, but was rapidly destroyed in an alkaline reaction and at 100°C.

The only report of a substance active upon actinomycetes and not upon true bacteria or fungi was made by Szabo and Marton (1955), who isolated from a culture of an actinomycete an "anti-actinomycete factor" that was highly active against actinomycetes, without any activity upon gram-positive and gram-negative bacteria.

Effect of Actinomycetes upon Saprophytic Soil Bacteria

The effect of actinomycetes upon the growth of soil saprophytes and upon plant pathogens has also received considerable attention. Nikolaieva first observed, in 1914, that actinomycetes may exert a repressive effect upon the growth of *Azotobacter*. Nickell and Burkholder (1947) studied the inhibition of growth of *Az. vinelandii* by various actinomycetes isolated from soil. When cultures of actinomycetes were mixed with soil samples and plated out, the nitrogen-fixing bacteria were either greatly reduced in number or completely eliminated. It was suggested that this serves to illustrate the importance of microbial antagonism in ecological investigations.

Another antagonistic effect of actinomycetes upon nitrogen-fixing bacteria may prove to be of even greater economic importance. Konishi and Fukuchi have shown that certain actinomycetes are able to inhibit the growth of root-nodule bacteria; some of the cultures, like *S. flavus*, were particularly effective.

Babak (1958) tested the sensitivity of 60 *Azotobacter* cultures to various species of *Streptomyces* and to various antibiotics, such as penicillin, streptomycin, gramicidin, and chlortetracycline. They were all found to be susceptible to the antibiotics. Their sensitivity to the *S. coelicolor* group was dissimilar; some strains proved inhibited and others indifferent.

When the antimicrobial properties of actinomycetes became well recognized, it was only natural that attempts should be made to encourage the development of these organisms in the soil, in order to depress the growth of various organisms capable of causing plant diseases.

Activity of Actinomycetes upon Plant Pathogenic Fungi

An extensive literature has accumulated upon the antagonistic effects of actinomycetes upon fungi, especially upon plant pathogens. References to such effects are reported earlier in this chapter. Winter presented (1949) further evidence concerning the ability of various actinomycetes to attack *Ophiobolus graminus*, an important parasite that attacks wheat. Sanford and Cormack tested the effect of eight cultures of actinomycetes upon the disease-producing fungus *Helminthosporium sativum*. In comparison with a disease rating of 66 per cent for the untreated pathogen, four actinomycetes depressed the virulence of the pathogen to 33, 22, 22, and 1 per cent, respectively; two had no marked effect; and the other two appeared to increase the virulence by 12 and 16 per cent, respectively.

Perrault demonstrated that the growth of *Colletotrichum sepedonicum* in agar media was impeded by several microorganisms isolated from potato tubers affected with ring rot. Four of these organisms were actinomycetes and were able to produce antibiotic substances that diffused readily through the medium and prevented all growth of the pathogen. One culture produced a lysis of the plant pathogen.

McGahen treated soils in the sugar-cane belt of Louisiana with bagasse compost, with cowpea and soybean trash, and with bloodmeal, tankage, bonemeal, and ammonium sulfate. The actinomycete populations were measured by the dilution methods; the antibiotic activities were determined by the degree of inhibition of *Pythium arrhenomanes* in culture. Nitrogen-rich materials, such as cowpea and soybean trash, bloodmeal, and tankage, gave marked increases in the actinomycete populations. The antibiotic index was increased by the use of bagasse compost and cowpea-soybean trash; a decrease of the antibiotic index occurred, however, when the soil was treated with bonemeal, bloodmeal, or tankage. Ammonium sulfate did not materially affect either the population or the antibiotic index. It was suggested that nitrogen-rich materials, like bloodmeal and tankage, cause an increase of the nonantagonistic and/or weakly antagonistic actinomycetes in favor of the moderately and highly antagonistic forms. The cellulosic materials cause an increase of the antagonistic over the nonantagonistic actinomycetes.

Antagonistic Effects of Fungi and Bacteria upon Actinomycetes

In a natural environment, such as soil, the antagonistic properties of actinomycetes, if they develop at all, will be exerted largely in an aerobic environment. Under anaerobic or microaerophilic conditions, the actinomycetes themselves may be antagonized; bacteria, like *Ps. fluorescens*, have been shown to exert a marked antagonistic action upon actinomycetes, causing their lysis. *B. megaterium* can also be antagonistic to certain species of actinomycetes, but also can be antagonized by others. The effect of bacteria upon the potato scab organism was studied in detail by Kieszling, who was able to prevent scab development in the soil by the use of such bacterial cultures.

Numerous fungi are also capable of exerting a marked destructive effect upon actinomycetes. This is true, for example, of the effect of the fungus antibiotic penicillin upon human and animal diseases caused by actinomycetes.

Are Antibiotics Produced in the Soil?

In 1945, Waksman presented evidence that it is highly doubtful that antibiotics are produced in the soil itself or that this phenomenon is of any great significance in modifying the microbiological population of the soil. The evidence was based upon the following observations:

(1) The fact that an organism produces an antibiotic in artificial culture is no evidence that is is capable of doing so in soil, particularly since relatively small changes in a nutrient medium may fundamentally affect the production of antibiotics in pure culture.

(2) Many known antibiotics are extremely unstable and could not be expected to remain unchanged in soil for sufficient time to have any effect.

(3) There is no evidence that production of antibiotics affects in any way the survival of organisms producing them.

(4) The fact that the organisms found in the soil possess no greater resistance to particular antibiotics than comparable strains found in other substrates add further weight to the non-existence of such antibiotics in soil, in concentrations sufficient to exert an effect.

Brian, Krassilnikov, and others argued against these assumptions. Brian emphasized that some antibiotics are very stable, that they can be produced locally, where they can have a maximum effect. Siminoff and Gottlieb (1951, 1952) could demonstrate the formation of antibiotics, notably streptomycin, in sterile soil but not in fresh soil. Pramer and Starkey established that streptomycin is rapidly destroyed in the soil.

Some recent evidence that antibotics are produced in soil has been submitted by Stevenson (1956). In most cases, however, the assumption that the occurrence of streptomyces capable of producing antibiotics will lead to the formation of such antibiotics in soil, and that this will lead to the elimination of pathogenic bacteria is farfetched. Such assumptions have been made, for example, for the elimination of typhus abdominalis bacteria in irrigated soils, merely because several streptomyces have been isolated from such soils.

Soil Enrichment with Pathogenic Organisms

Soil enrichment with various chemical compounds results in the isolation of organisms capable of bringing about certain specific reactions. This is true, for example, of the development of organisms capable of decomposing cellulose or pectin by the addition of these substances to the soil; it is also true for the enrichment of soil with substances like sulfur and ammonia to encourage the development in the soil of sulfur-oxidizing and nitrifying bacteria. It was at first believed that the same principle would apply to the isolation of antibiotic-producing organisms. Claims were actually made that favorable results were obtained. But the introduction into the soil of cultures of microorganisms led to their destruction (Katznelson, 1940).

Waksman and Woodruff (1940) attempted to encourage by soil enrichment the development of antibiotic-producing actinomycetes. They at first believed this had succeeded. They said that the survival of bacteria added to the soil, but not indigenous to it, resulted in the rapid dying out of such added bacteria. This was believed to have been accompanied by an increase in the numbers of soil bacteria and actinomycetes capable of developing on the plate. On further additions of cultures of bacteria to the soil, more rapid destruction of the added organisms resulted. The nature of the antagonists developing in the soil depended upon the bacteria added, the soil treatment, and the temperature of incubation. Several antagonists were isolated from the soil. Sterile culture filtrates of these antagonists were capable of reducing considerably the numbers of bacteria and actinomycetes in the soil. A highly active substance was obtained from a soil actinomycete.

TABLE 58

Growth inhibition of streptomyces by their respective antibiotics (Waksman, Reilly, and Johnstone)

Nature of antibiotic	Organism producing antibiotic	Activity of preparation per 1 gm	Dilution units per mg, expressed as activity against		
			S. antibioticus	*S. lavendulae*	*S. griseus*
Actinomycin	*S. antibioticus*	100,000*	100	5,000	100
Streptothricin	*S. lavendulae*	100†	1,000	0.4	10
Streptomycin	*S. griseus*	125†	1,000	100	1.2

* *S. lutea* units; crystalline material.
† *E. coli* units; crude preparations.

TABLE 59

Cross-resistance among different streptomyces (Krassilnikov)

Test organisms	Antagonist						
	S. violaceus	*S. coelicolor*	*S. ruber*	*S. griseus*	*S. globisporus*	*S. roseus*	*S. albus*
S. violaceus	–	+	+	+	+	+	–
S. coelicolor	+	–	+	+	+	+	–
S. ruber	+	+	–	+	+	+	+
S. griseus	+	+	+	–	+	–	–
S. globisporus	+	+	+	+	–	+	+
S. longisporus	+	+	+	+	+	–	+
S. roseus	+	–	+	+	+	–	+
S. albus	–	–	–	+	–	+	–

Further studies (Waksman and Schatz, 1946), however, on the enrichment of soil with suspensions of living gram-negative bacteria and with both living and dead tuberculosis organisms resulted in complete failure to isolate cultures of actinomycetes that might possess specific activity against the organisms added to the soil.

The writer received numerous suggestions that he use soils from cemeteries in which sufferers from tuberculosis and other infectious diseases or cancer victims were recently buried. He can do no better than to quote from Routien and Finlay, who followed up such suggestions. They attempted on two occasions to isolate antagonistic organisms from such sources. Soil from an orthodox Jewish cemetery, where bodies were interred soon after death without embalming, was examined for antagonists against the pathogens introduced into the soil with the diseased body.

Soil samples and earthworms were dug from the graves, but the cultures obtained "were disappointingly devoid of interesting antagonists." Soil samples were also taken along the route of a sewage effluent from a sanatorium for tuberculous patients. "Samples of the soil taken along this channel of treatment from the raw sewage to a point 50 yards downhill yielded no organism of high antagonistic powers."

TABLE 60

Cross-resistance of antibiotic-producing actinomycetes (Teillon)

Original streak*	Secondary streaks							
	Theoretical results				Experimental results			
	A	B	C	D	A	B	C	D
A	0	+	+	+	0	0	0	+
B	+	0	+	+	0	0	+	+
C	+	+	0	+	+	+	0	+
D	+	+	+	0	+	+	+	+

* A = *S. rimosus*; B = *S. griseus*; C = *S. aureofaciens*, D = *S. fradiae*.

Cross-resistance of Microorganisms as Diagnostic Criteria

Waksman, Reilly, and Johnstone (1946) first demonstrated that actinomycetes are, as a rule, resistant to the antibiotics produced by them (Table 58). However, some organisms tended to be less sensitive to certain antibiotics than to others. These investigators suggested taking advantage of this phenomenon for the isolation from the soil of specific organisms producing a particular antibiotic, and for isolation of more potent antibiotic-producing strains from a mixed mother culture. It was later shown (Waksman and Lechevalier) that certain organisms, like *S. fradiae*, are sensitive to their own antibiotics. Krassilnikov accepted the phenomenon of cross-resistance within the species of actinomycetes as offering a de-

TABLE 61

Cross-streak tests with 12 strains of S. fradiae *on yeast-glucose agar* (Waksman *et al.*, 1958)

Original streak	Zones of inhibition, mm											
	3535	3554	3556a	3556b	3572	3594	3719	3566	3595	3598	3596	3597
3535	5.0	1.5	0	3.5	3.0	0.5	1.0	3.5	3.0	3.5	1.0	0
3554	1.0	1.3	1.0	1.0	2.3	0.7	1.0	1.6	1.0	2.0	0.7	0.7
3556a	2.3	1.3	3.0	2.6	3.0	3.3	5.0	1.6	2.3	1.6	0.6	1.0
3556b	1.6	2.6	0.6	1.0	3.3	1.6	4.0	2.6	2.3	3.0	4.0	0
3572	1.0	0.3	1.6	2.5	2.0	2.0	1.0	2.3	1.6	2.0	0.6	0.6
3594	1.3	1.3	0.6	1.0	2.6	1.6	2.0	2.0	1.6	1.3	0.6	0.3
3719	4.0	4.5	4.8	4.5	4.0	4.0	3.0	5.0	4.0	6.0	3.0	1.5
3566	12.6	14.3	11.6	12.6	14.0	14.6	4.0	1.0	2.3	3.0	1.6	3.3
3595	15.6	16.6	14.6	12.3	16.6	16.6	7.0	0.6	0.3	0.6	0	0
3598	16.0	17.0	11.6	16.0	22.6	18.0	17.0	0.6	0	2.0	0	0
3596	4.0	4.3	3.3	3.6	5.3	6.0	4.0	0	0.6	0	0	0
3597	9.3	10.6	11.6	10.6	10.6	9.0	7.0	3.3	1.0	0	0	0

sirable property for species differentiation (Table 59). Umezawa, Oki and Hata, Kuroya, and others also accepted this concept for classifying actinomycetes.

Teillon (1952), who made a detailed study of the cross-resistance of actinomycetes producing antibiotics, found various important exceptions to the principle expostulated by Kuroya and Krassilnikov (Table 59). On the basis of these results, he expressed doubt concerning the validity of the claim of the Japanese investigators that "the cross-inhibition test is a useful and easy method of differentiation." The method of cross-inhibition did not appear to Teillon as sufficiently reliable for differentiating streptomyces cultures and their antibiotics. The organisms were shown to form a variety of metabolic products that could readily mask the results. Further, some organisms produced certain other antibiotics that, although in themselves possibly not quantitatively significant, modified the effects of the major antibiotics produced by the organisms. Some strains related to *S. griseus* were able, for example, to produce streptothricin as well as streptomycin. Phenomena of autoinhibition are also not uncommon among actinomycetes, such as *S. aureofaciens* and *S. fradiae.*

Finally, the quantitative variations in the production of antibiotics do not permit the laying down of hard and fast rules concerning the possible effect of cross-inhibition under natural conditions. This is brought out in Table 61 on the auto- and cross-inhibition of various neomycin-producing and other strains of *S. fradiae*. It is possible to observe occasionally certain self-inhibition of growth by various actinomycetes, especially by pigment-forming types. This has been ascribed by Krassilnikov *et al.* (1958) to special substances of an enzymatic nature designated as "necrohormones."

CHAPTER 15

Production of Antibiotics

Isolation of Antibiotic-producing Cultures

The actinomycetes occupy a leading place among the antibiotic-producing groups of microorganisms. Already, they have yielded nearly 500 compounds and preparations, including some of the most important chemotherapeutic agents now known. Some of these antibiotics are active only on bacteria, others only on fungi; some are active on viruses and phages, and others are active on tumors. Some of the antibiotics are said to be of a broad spectrum type, able to repress the growth of both bacteria and fungi, or of bacteria, rickettsiae, and the larger viruses. Some have a narrow antibiotic spectrum, such as those that are largely active against gram-positive bacteria or mycobacteria, or yeast-like organisms (Waksman, 1955).

Among the antibiotic-producing actinomycetes, the genus *Streptomyces* occupies a leading place. Certain groups of this genus, like *S. griseus* and *S. lavendulae*, are characterized by the formation of a large number of different antibiotics. Some antibiotics or closely related groups of antibiotics (antibiotic complexes) are produced by different organisms. This is true, for example, of the streptomycin and neomycin complexes. There are also marked quantitative variations in the production of antibiotics by different strains of the same organism. Composition of medium and environmental conditions play an important part in this connection.

A number of the antibiotics produced by actinomycetes have been isolated in a pure state, their chemical structures established, and their antimicrobial properties studied in detail. Other antibiotics have been described only as preparations, or in such a preliminary manner that it is not certain whether one is dealing with a single entity or with a group of closely related chemical compounds. Only one of the actinomycete antibiotics, chloramphenicol, has been synthesized. Others have been modified chemically to give compounds with slightly different types of activity. This is true of the formation of dihydrostreptomycin from streptomycin and of tetracycline from chlortetracycline. Definite chemical structures have been established for a number of antibiotics, notably the streptomycins, cycloheximide, the actinomycins, the tetracyclines, cycloserine, azoserine, erythromycins, and sarkomycin. These were found to comprise a variety of highly interesting organic structures, some of which have never been known before.

Principles of Antibiotic Production

Certain general principles concerning the production of antibiotics by actinomycetes have been established:

1. Different strains of an antibiotic-producing species may form several antibiotics that are not related chemically: *S. griseus*

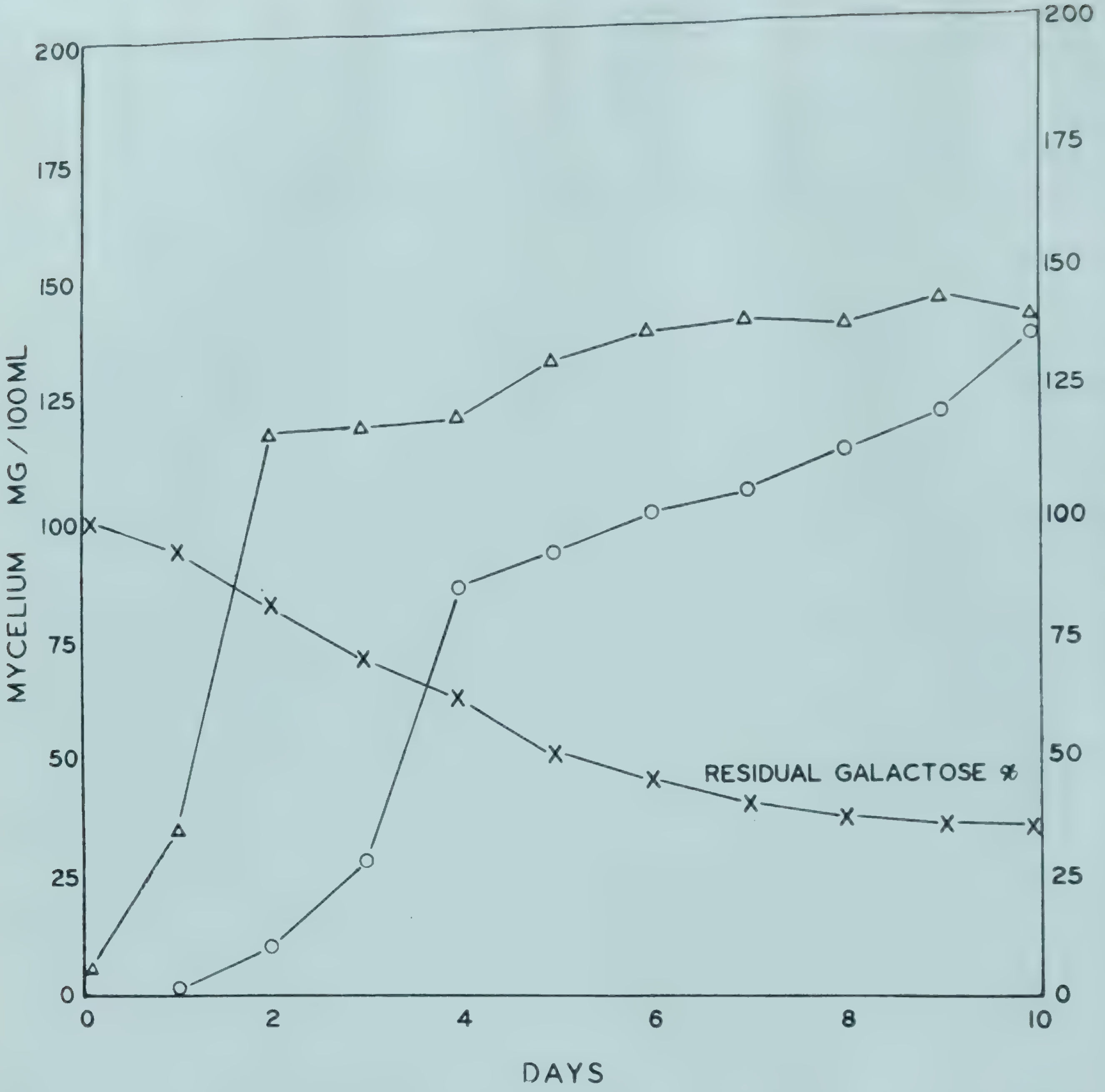


FIGURE 85. Utilization of galactose by *S. antibioticus* for growth and actinomycin production: △——△ = dry weight of mycelium in mg per 100 ml of medium; ×——× = percentage of residual galactose; ○——○ = actinomycin produced, µg/ml of medium (Reproduced by special permission from: Katz, E., Pienta, P., and Sivak, A. Appl. Microbiol. 6: 238, 1958).

strains produce streptomycin, streptocin, cycloheximide, and grisein; *S. fradiae* strains produce neomycins and fradicin; *S. rimosus* produces oxytetracycline and rimocidin.

2. A single strain of an antibiotic-producing organism may form several chemically related antibiotic substances. This is true of the production of mannosidostreptomycin and streptomycin by *S. griseus*; of neomycin B, and C by *S. fradiae*; of viomycin A, B, and C by *S. vinaceus*.

3. Organisms producing the same antibiotic or closely related compounds are found in different soil regions throughout the world. This is true, for example, of cultures of streptomyces producing actinomycin, streptomycin, chloramphenicol, the tetracyclines, the erythromycins, and others. Since the strains

producing the same antibiotic may vary, the morphological criteria alone cannot be used as a basis for identifying antibiotics, or the formation of a particular antibiotic for identification of species.

4. A change in the nutrition of the organism may result in a change in the nature of the antibiotic produced. This is true, for example, of the formation of the various actinomycins.

5. As a rule, antibiotic-forming organisms are resistant to the antibiotics they produce, as shown in Chapter 14. This phenomenon is taken advantage of in the isolation of fresh cultures capable of forming a given antibiotic, and in the selection, from a given culture, of more potent strains. Not all antibiotic-forming organisms, however, behave in the same manner.

Methods for Isolating and Testing Antibiotic-producing Organisms

In a search for antibiotic-producing cultures of actinomycetes, certain steps are usually followed:

1. A sample of soil is plated out on suitable agar media. Usually, simple synthetic or poor organic media are selected to encourage the maximum development of colonies but prevent these from making heavy growth, thus avoiding overcrowding.

2. After a given period of incubation, which may vary from 3 to 15 days, the colonies of the actinomycetes developing on the plates are picked and transferred to fresh agar slants. If care is taken in making the transfer, pure cultures are usually obtained. Otherwise, the cultures may have to be purified by replating and reisolating.

3. The cultures thus obtained are tested, by the agar-streak method, for their ability to inhibit the growth of microorganisms. Various bacteria, comprising gram-positive and gram-negative forms, and fungi are commonly used as test organisms. In some cases, especially in search for antiviral or antitumor agents, more complicated procedures are employed. Such cultures of actinomycetes as are found to possess the desirable antimicrobial properties are selected for further study.

4. The selected cultures are grown in various liquid media, in stationary or under submerged conditions, for varying periods (usually 3 to 10 days), and the antibiotic spectra of the broths determined.

5. Suitable media and proper growth conditions are established for each individual culture. The nature of the medium for the production of the antibiotic is of great importance; each culture may require special media for optimum production of the antibiotic.

6. An effort is then made to isolate from the medium the active substance produced by the culture, concentrate it, and purify it.

7. The isolated antibiotic is studied for its chemical, physical, and antimicrobial properties. A comparison is also made of its antibiotic spectrum, which should correspond to that of the culture broth from which it was isolated. A lack of proper correlation may be due either to the presence of more than one antibiotic in the broth, or to a chemical modification of the antibiotic in the process of purification.

8. The isolated antibiotic is tested for toxicity and activity in experimental animals.

9. Before actual production of the antibiotic is undertaken, the culture is irradiated or treated by other suitable procedures, and the isolated colonies retested, since a freshly isolated culture may not be a very potent one. A search for the natural occurrence of more potent strains is often made.

10. The clinical evaluation of the isolated antibiotic is the final step in the group of procedures. This permits one to come to a conclusion concerning the therapeutic potentialities of the freshly isolated antibiotic.

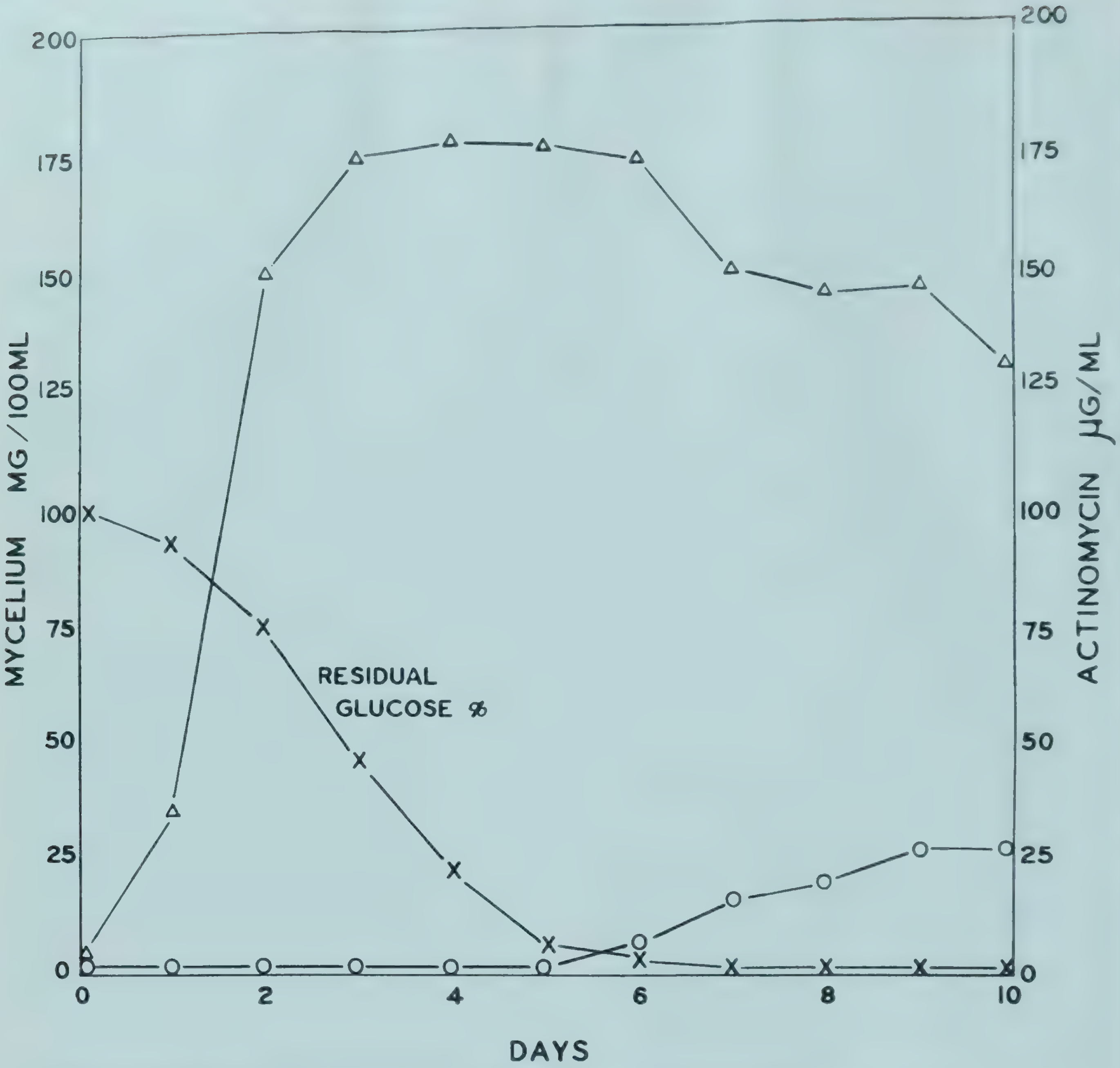


Figure 86. Utilization of glucose by *S. antibioticus* for growth and actinomycin production: △——△ = dry weight of mycelium in mg per 100 ml of medium; ×——× = percentage of residual glucose; ○— —○ = actinomycin produced, µg/ml of medium (Reproduced by special permission from: Katz, E., Pienta, P., and Sivak, A. Appl. Microbiol. **6:** 237, 1958.)

Numerous comprehensive reviews have been published on the production of antibiotics by actinomycetes (Waksman *et al.*, 1946; Benedict, 1953; Krassilnikov, 1950; Waksman and Lechevalier, 1953).

Antimicrobial Spectra

Differences in the range of antimicrobial activities of various antibiotics are both quantitative and qualitative in nature. The antimicrobial spectrum of each antibiotic is so characteristic in nature, when determined under standard conditions and with standard test organisms, that it can be used for the determination of its particular specificity. The antibiotics can be grouped not only on the basis of their chemical properties, but also upon the basis of the specificity of their corresponding spectra.

Streptomycin, streptothricin, and neomycin show similar antibiotic spectra and can, therefore, be grouped together. They are all basic antibiotics, soluble in water and active against gram-positive, gram-negative, and acid-fast bacteria; they are not active upon viruses. They show marked differences, however: Streptothricin is active on a large number of fungi and inactive on *B. cereus*. Streptomycin is active on only very few fungi, mostly phycomycetes, and on *B. cereus*. Neomycin is not active on fungi, but is active on *B. cereus*. These three antibiotics vary in the degree of their activity on individual bacterial species and strains; they also differ in their toxicity to animals. These antibiotics each represent a group or a complex of closely related chemical substances, the individual components showing differences in their antimicrobial activities, which are largely quantitative in nature.

The same relationships and differences are found in the tetracycline group of antibiotics.

Some antibiotics are characterized by very narrow antimicrobial spectra: Viomycin is active chiefly against *M. smegmatis* and certain other mycobacteria. Other antibiotics have wide spectra, such as chloramphenicol and the tetracyclines, which are active not only upon gram-positive and gram-negative bacteria, but also upon rickettsiae and the larger viruses.

TABLE 62

Comparative antibiotic spectra of streptomycin and streptothricin

On basis of crude, ash-free dry material

Organism	Gram stain	Units of activity per milligram of ash-free dry material	
		Streptomycin	Streptothricin
B. subtilis	+	500	500
B. mycoides	+	1000	<3
B. cereus	+	120	<3
B. megaterium	+	400	150
Staph. aureus	+	60	200
Sar. lutea	+	400	150
M. phlei	+	400	50
M. tuberculosis	+	120	—
Ph. pruni	−	400	400
E. coli	−	100	100
Serr. marcescens	−	100	5
Aer. aerogenes	−	40	50
Pr. vulgaris	−	40	50
Ps. fluorescens	−	8	<3
Ps. aeruginosa	−	4	<3
Cl. butylicum	−	12	<3

The development of resistance among sensitive bacteria against specific antibiotics and the problems of cross-resistance among the different antibiotics can also be utilized for the purpose of classifying and identifying antibiotics.

Production of Antibiotics

For the production of antibiotics, complex organic media, containing yeast extract, soybean meal, meat extract, or similar materials, are usually required. Synthetic media can also be used. O'Brien *et al.* (1952) demonstrated that as good yields of streptomycin can be obtained on a proper synthetic medium as on a complex soybean meal-glucose medium. Such a synthetic medium consists of glucose, glycine, sodium acetate, magnesium sulfate, potassium phosphate, and traces of iron, zinc, copper, calcium, and manganese. The acetate can be replaced by glutamate or succinate.

For maximum yields, however, specific media and specific conditions must be developed, not only for each antibiotic, but for each strain of each organism producing a given antibiotic.

Classification of Antibiotics

Various systems have been proposed for the classification of antibiotics of actinomycetes. Kurosawa suggested the combination of the ability of different organisms to utilize specific carbohydrates and to form antibiotics as a basis for the classification of actinomycetes. Six groups of actinomycetes were thus recognized:

I. Those organisms that are rhamnose- and raffinose-negative, but are xylose-, mannitol-, and lactose-positive. The various streptomycin- and streptothricin - producing strains belong to this group.

II. Those organisms that show the same type of carbon utilization. These include strains of *S. griseus*, *S. olivaceus*, and *S. poolensis*.

Groups III–VI comprised mostly streptothricin-, chloramphenicol-, and tetracycline-forming groups.

A combination of chemical, physical, and biological properties of the antibiotics produced by actinomycetes can be utilized in developing a proper system of classification, as will be shown in Chapter 32 (Volume III). At present, some 20 different categories of antibiotics may be recognized. Some of these are the following:

1. The actinomycins and other polypeptides.
2. The glucosidic antibiotics.
3. The macrolides.
4. The pigmented antibiotics.
5. Chloramphenicol.
6. The tetracyclines.

TABLE 63

Antibacterial activities of the various streptomycins (Rake *et al.*)

Test organism	Minimal inhibiting concentration,* μg/ml			
	Streptomycin	Dihydrostreptomycin	Mannosidostreptomycin	Dihydromannosidostreptomycin
K. pneumoniae (ATCC 9997)	1.76	1.76	6.39	6.59
Aer. aerogenes (ATCC 129)	2.71	3.27	10.80	11.10
E. coli (D 56)	6.05	6.79	24.80	23.80
Sal. schottmülleri (D 51)	10.10	36.50	14.30	14.40
Sal. typhosa (D 15)	12.20	51.00	12.40	12.90
Sal. enteriditis (D 61)	4.14	5.50	12.70	13.60
Sh. sonnei (H 1414)	7.42	8.52	30.60	30.30
Sh. dysenteriae (H 141)	6.26	5.82	27.20	27.10
Br. abortus (Huddleson 1119 avirulent)	0.816	0.738	2.93	2.53
H. influenzae type b (D 68)	2.30	1.53	8.53	5.53
M. pyogenes (*Staphylococcus*) var. *aureus* (209P)	0.828	1.39	5.64	7.77
Staph. pyogenes (C203)	11.70	15.90	82.90	87.90
Mycobacterium tuberculosis:				
H37Rv	2.00	2.20	5.50	6.50
Ravenel	0.58	0.62	2.50	2.20
BCG	0.52	0.55	1.90	1.70
N†	0.54	0.56	2.50	2.10
T†	0.55	0.54	2.20	2.00
P†	0.62	0.85	2.30	2.20
OD†	0.63	0.75	2.30	2.60
K†	1.00	1.70	3.90	3.90

* All figures are given in terms of weight of the trihydrochlorides. On the basis of assays with *K. pneumoniae*, the streptomycin and dihydrostreptomycin would have an activity of 820 units per mg, the mannosidostreptomycin an activity of 236 units per mg, and the dihydromannosidostreptomycin 228 units per mg.

† Strains of *M. tuberculosis* freshly isolated from human cases.

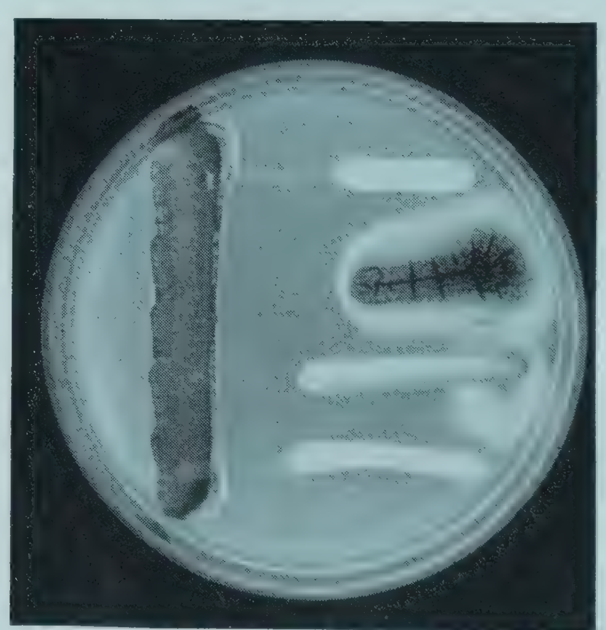


FIGURE 87. Antibiotic spectrum of *S. fradiae* (top to bottom: *Saccharomyces cerevisiae, Aspergillus niger, Serr. marcescens, Micrococcus lysodeikticus*).

7. Grisein and other iron-containing antibiotics.
8. Sulfur-containing antibiotics.
9. The polyenes.
10. The nonnitrogenous substances.
11. Antitumor and antiviral agents.
12. Various unidentified antibiotics.

Some of these groups, because of their historical significance, may now be discussed in greater detail.

The Actinomycins

The first representative of the actinomycins was isolated and crystallized in 1940 by Waksman and Woodruff. It was produced by an organism described as *S. antibioticus.* Its chemical nature was studied by Waksman and Tishler, and it was designated as actinomycin A. Although highly effective against different microorganisms, it proved to be too toxic to experimental animals (Robinson and Waksman). Another representative of this group was isolated by Lehr and Berger; it was studied by Dalgliesh and Todd (1949–1952), who designated it as actinomycin B. In 1949, Brockmann and Grubhofer (1949–1953) isolated a third form of actinomycin (C) from a culture of *S. chrysomallus.* (See also Brockmann, 1954; Brockmann and Gröne, 1954; Brockmann and Muxfeldt, 1954–1956). Still other actinomycins were isolated later, in various parts of the world, notably actinomycin D by Waksman and Gregory from a culture of *S. parvullus* (see Vining *et al.*, 1954; Johnson, 1956; Bullock and Johnson, 1957), actinomycin I and X (Brockmann *et al.*), J (Hirata and Nakanishi), M, and others. Some related compounds, like actinoleucin, have also been isolated (Ueda *et al.*). It appeared that in every new screening program of actinomycetes, actinomycin was the first antibiotic obtained (Waksman *et al.*, 1946; Welsch *et al.*, 1946).

Although nearly all actinomycins are produced by streptomyces, some are also formed by certain micromonosporas (Fisher *et al.*).

Table 64

Effect of carbohydrates as carbon sources on actinomycin production by S. antibioticus (Katz, Pienta, and Sivak)

Carbohydrate, 1.0 per cent	Maximum actinomycin assay, μg/ml
L(+) Arabinose	86
D(−) Arabinose	0
D(+) Xylose	79
D-Ribose	0
D-Glucose	20
D-Galactose	124
D-Fructose	25
D-Mannose	10
L-Rhamnose	49
Lactose	24
Maltose	41
Sucrose	0
Cellobiose	14
Sorbose	0
Raffinose	0
Dextrin	43
Starch	11
Glycogen	16
Inulin	0

The composition of the medium was found to exert a marked effect upon the composition of the actinomycin molecule (Schmidt-Karsten, Goss and Katz). Sevcik (1957) has shown that the production of actinomycin by *S. antibioticus* depends on the amount of phenol oxidase (laccase type) present in the mycelium and decreases with the pH of the medium, the optimum being pH 5.0. Certain soil bacteria are capable of producing an enzyme which has the capacity of inactivating actinomycin preparations (Katz and Pienta, 1957).

Each form of actinomycin, produced by different organisms or under different conditions of culture, was found to be a heterogeneous compound, made up of several individual chemical entities. The molecule consists of a chromophore nucleus to which are attached several amino acids. The nature of these actinomycins and their individual components depend on the makeup of the polypeptide part of the molecule. Waksman,

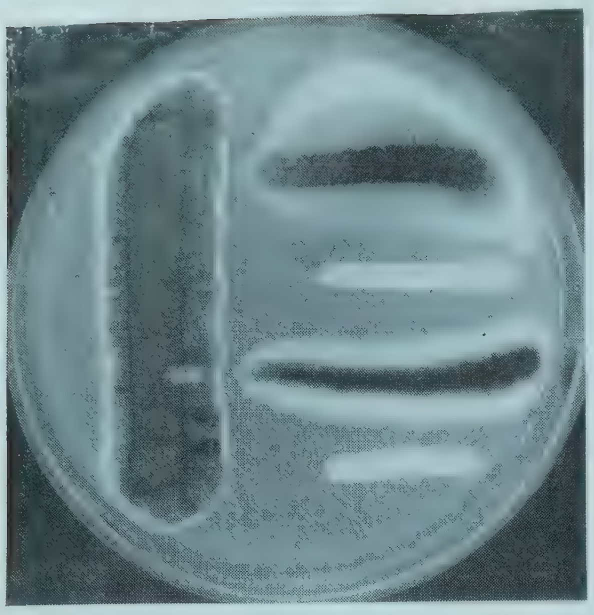


FIGURE 88. Antifungal activity of a streptomyces. The two filamentous fungi are resistant and the two yeasts are sensitive.

Katz, and Vining (1958) proposed a system for classifying the various actinomycins, based on their chemical structure. The paper chromatographic methods are very convenient for their differentiation.

Among the interesting developments in connection with the study of the actinomycins is their effect upon neoplastic growths. The work of Hackmann, begun in 1952, on the effect of actinomycin C on experimental tumors and the clinical observations of Schulte and Lings (1953) opened a new field for the potential utilization of this group of antibiotics as chemotherapeutic agents. These results were soon confirmed by a number of clinical investigators. It is sufficient to mention the studies of Trounce *et al.*, Sigiura and Schmidt, Nitta *et al.*, Yamashita *et al.*, and Farber and Burchinal (1958). These studies brought out the fact that although different actinomycins may vary somewhat in their toxicity, they are similar in their cytostatic activity (Pugh *et al.*, 1956). Foley reported his observations on the mechanism of the action of actinomycin in bacterial systems (For a review of the literature, see Reilly, 1953; Waksman, 1954; and Hackmann, 1955).

The Basic Antibiotics

Streptothricin

The first basic antibiotic was isolated from a culture of *S. lavendulae* by Waksman and Woodruff in 1942. They named it streptothricin, thus honoring F. Cohn's first designation of an actinomycete culture. It was found to possess highly desirable chemical and biological properties and it offered promise of becoming an important chemotherapeutic agent. It was water-soluble and thermostable. It was active against various gram-positive and gram-negative bacteria and fungi. It was less toxic than actinomycin, but more toxic than penicillin, an antibiotic that had come to occupy an important place in chemotherapy. Its delayed toxicity prevented its immediate clinical use (Waksman, 1943).

The *in vivo* activity of streptothricin was first established by Metzger *et al.* (1942); its action upon the tuberculosis organism was later demonstrated by Woodruff and Foster (1944). It was later crystallized by Fried and Wintersteiner (1945), and by Kuehl *et al.* (1945–1946). The search for streptothricin-like substances continued for a long time, partly because of their antituberculosis properties (Weiser *et al.*) and partly because of their intriguing chemistry. Numerous related compounds were described under a variety of different names.

Numerous other preparations were later isolated from cultures of *S. lavendulae* (Bohonos *et al.*). Some were found to be the same as streptothricin, and others were either closely related or comprised mixtures of different antibiotics.

Soon after the isolation of streptothricin, another antibiotic, designated as proactinomycin, was isolated (Gardner and Chain,

1942) from a culture of an organism believed to be a nocardia (proactinomyces) but now recognized as a streptomyces. Its antibacterial and other biological properties were later established by Florey *et al.* (1945) and by Marston and Florey.

Streptomycin

The experience gained in the study of the formation and isolation of streptothricin from cultures of actinomycetes proved to be highly important in planning a search for other antibiotic agents that would possess similar or even more desirable biological and chemical properties, such as a broad antibiotic spectrum and a lesser toxicity to the animal body. After further extensive studies of many actinomycetes representing a great variety of species and varieties, two cultures were found to yield an important antibiotic. These cultures were isolated from the soil and from the throat of a chicken. They both belonged to a species described in 1916 as *Actinomyces griseus,* the first representative of which was isolated by Waksman and Curtis in 1916 from the soil. The generic name of the organism was changed by Waksman and Henrici in 1943 from *Actinomyces* to *Streptomyces.* To honor this new generic name, the new antibiotic was designated as streptomycin.

As previously noted, the two cultures of the streptomycin-producing organism were first isolated in September 1943. Because of the similarity of the new antibiotic to streptothricin, both in isolation procedures and in its antibiotic spectrum, rapid progress was made in the development of suitable methods for the growth of the organism *S. griseus,* for the isolation of streptomycin, and for the evaluation of its antimicrobial properties. In January 1944, four months after *S. griseus* was isolated, the isolation of streptomycin was announced by Schatz, Bugie, and Waksman.

Special methods were soon developed for the isolation of other streptomycin-producing cultures, as well as for obtaining more potent strains from the mother culture, by using media containing varying concentrations of streptomycin (Waksman, Bugie, and Schatz; Waksman, Reilly, and Schatz). Streptomycin was the first antibiotic produced by actinomycetes that took a prominent place in the treatment of numerous infectious diseases in man, animals, and plants. It also proved to be the first drug effective against the Great White Plague of man, tuberculosis, as first shown by Schatz and Waksman (1944).

The activity of streptomycin upon bacterial infections in experimental animals was first established by Jones *et al.* (1944), and in experimental tuberculosis by Feldman and Hinshaw (1944), and later by Youmans and McCarter (1945), and Smith and McCloskey (1945). The effectiveness of streptomycin in clinical tuberculosis was first established by Hinshaw and Feldman (1946), followed by Cooke *et al.* (1946), who treated the first case of tubercular meningitis with streptomycin, and soon by numerous others (Keefer *et al.*).

Streptomycin was soon isolated in crystalline form (Carter *et al.*, 1945; Peck *et al.*, 1945) and its chemical nature determined. In standardizing streptomycin (Waksman, 1945), it was found that 1 unit of the antibiotic, as determined by its activity upon a standard strain of *E. coli*, under standard conditions of culture, was equivalent to 1 μg of the pure base.

Gottlieb and Anderson have shown that the production of streptomycin by *S. griseus* is dependent on many factors. Among these are the constitution of the medium, hydrogen-ion concentration, temperature, oxygen supply, and agitation of the medium. The process of antibiotic formation followed the same general pattern in all the media. No

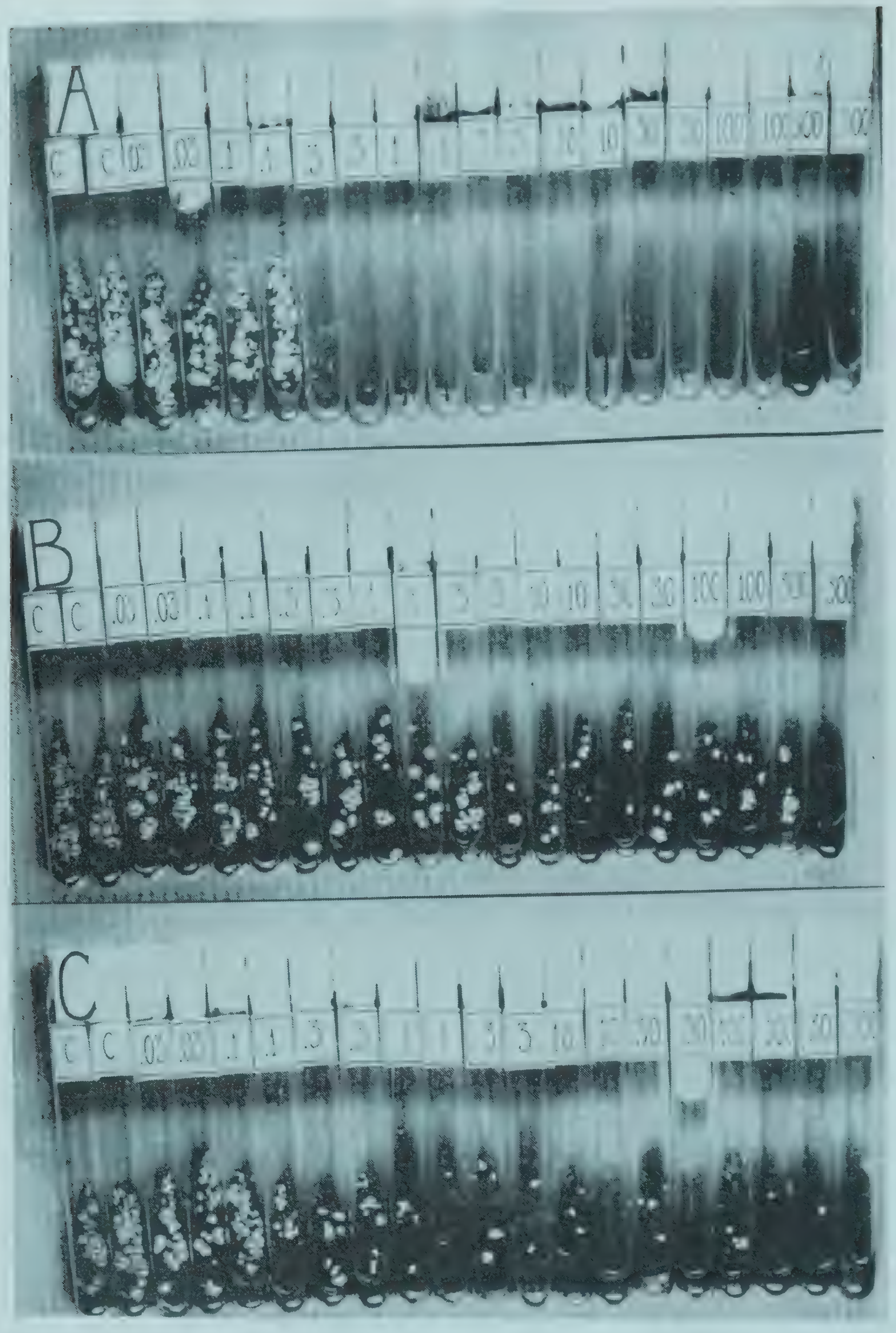

A
C C .03 .03 .1 .1 .3 .3 1 1 3 3 10 10 30 30 100 100 300 300
B
C C .03 .03 .1 .1 .3 .3 1 1 3 3 10 10 30 30 100 100 300 300
C
C C .03 .03 .1 .1 .3 .3 1 1 3 3 10 10 30 30 100 100 300 300

FIGURE 89. First experiment in which the effect of streptomycin upon the growth of *M. tuberculosis* was demonstrated (Reproduced from: Schatz, A. and Waksman, S. A. Proc. Soc. Exptl. Biol. Med. 57: 246, 1944).

streptomycin was detected in the solution during the first 12 hours of growth of the organism. The formation of the antibiotic increased steadily from then on until a maximum was reached between 48 and 60 hours of growth. After this, a decline in the streptomycin content of the solution occurred. This decrease continued until after about 96 hours, when the slope of the curve decreased with a tendency to level off. In all cases the peak of the streptomycin curve lagged behind the peak of the growth curve by about 24 hours. The conclusion was reached that streptomycin production in the medium is not primarily a function of the active growth phase of *S. griseus*. Only about 25 to 50 per cent of the total streptomycin had been released into the solution by the time the growth peak was reached.

On continued growth in artificial media, *S. griseus* may become contaminated with a phage or virus, designated as actinophage. The growth of the streptomycin-producing organism and the production of streptomycin are rapidly diminished. By the use of the plaque method, it is possible to measure the actual concentrations of actinophage in the culture. The number of particles per milliliter can reach as high as 10^{10}. Phage-resistant strains can be readily obtained from infected cultures.

Streptomycin is a strong base, belonging to the glucosides, in which a diguanido-group is linked to a nitrogen-containing disaccharide-like compound. A molecular weight determination on the trihydrochloride in water gave about 800 for the free base after the necessary corrections for the chloride ion.

Investigations carried out by Hunter *et al.* on *S. griseus* with the aid of $C^{14}O_2$ have shown that the carbons of the guanidine side chains in streptomycin are derived very largely, and possibly entirely, from carbon dioxide. The maximum incorporation of $C^{14}O_2$ into streptomycin was between 0.4 and 0.5 per cent. A much lower degree of fixation of C^{14} was obtained when no $C^{14}O_2$ was passed for the first 72 hours of the fermentation. L-arginine possibly functions as an intermediate in the biosynthesis of the guanidine side chains of streptomycin. A variety of compounds, either containing guanidine groups or readily changed into such compounds, are converted by *S. griseus* into a further substance containing at least one guanidine group. This compound has not yet been identified but may be involved in the biosynthesis of streptomycin by the organism.

On catalytic hydrogenation of streptomycin, two hydrogen atoms are added to the molecule, giving dihydrostreptomycin.

Recently it was reported that certain organisms (*S. humidus*) can produce dihydrostreptomycin directly in the medium. This product is similar to streptomycin in its antibacterial and pharmacological properties, except that in many cases it exerts a less severe effect upon vestibular dysfunction, although it may give greater autotoxicity. These results so far have not been confirmed.

Certain organisms belonging to the *Streptomyces* group also produce desoxystreptomycin, which was found to be more toxic than streptomycin.

Streptomycin is active against a large number of bacteria found among the gram-negative, gram-positive, acid-fast, and spirochaetal groups. Many of the bacteria affected by streptomycin are able to cause a great variety of human, animal, and plant diseases. Streptomycin is also active against certain plant-disease-producing fungi belonging to the phycomycetes. It is not active against anaerobic bacteria, protozoa, viruses, and the majority of fungi. The sensitivity of a given organism to streptomycin depends not only upon the species, but also upon the strain, and upon the composition of the medium in which it is tested. The bacteriostatic and bactericidal action of streptomycin upon *M. tuberculosis*, the causative agent of tuberculosis, is particularly significant. It is active in a concentration of 0.05 to 0.4 μg/ml. The bactericidal action also varies with concentration and the length of contact with streptomycin, 0.3 μg exerting an effect in 48 hours, and 20 μg/ml in 6 hours.

Sensitive bacteria become more resistant to streptomycin upon prolonged contact with the antibiotic. This is of considerable theo-

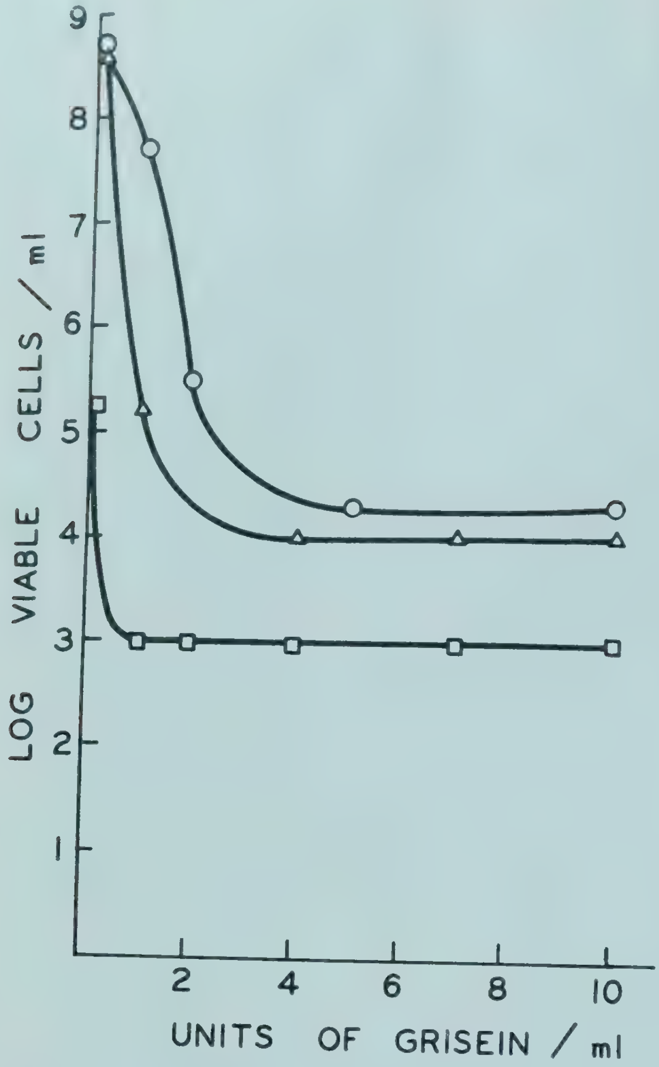


Figure 90. Effect of antibiotic concentration on development of resistance by *E. coli* in agar media: ○, Grisein alone; △, Grisein + streptomycin 1 μg/ml; □ Grisein + streptomycin 2 μg/ml. (Reproduced from: Reynolds, D. M. and Waksman, S. A. J. Bacteriol. **55**: 750, 1948).

The Macrolides

The first of these antibiotics (erythromycin) was isolated by McGuire *et al.* (1952) from a culture of *S. erythreus*. It is a basic compound, soluble in water, alcohol, and other organic solvents. It has significant activity against gram-positive organisms and some of the more important gram-negative bacteria, such as the *Neisseria*, *Hemophilus*, and *Brucella* groups; it is also active upon rickettsiae, the larger viruses, and some protozoa.

A number of other erythromycin-like compounds have been isolated and described under a variety of names, such as picromycin, magnamycin (carbomycin), oleandomycin, methymycin, and spiromycin.

Novobiocin

This antibiotic is produced by *S. niveus* and by *S. spheroides*. It has been isolated simultaneously in different laboratories and described under different names (streptonivicin, cathomycin, cardelmycin, vulcanomycin, etc.). It is highly active against *Staph. aureus*, as well as against a variety of other gram-positive and some gram-negative bacteria, but it causes allergic dermatitis.

Streptovaricin

This orange-red group of antibiotics, which contains at least five closely related components, was isolated from the culture of an organism described as *S. spectabilis*. It is active against various gram-positive, gram-negative, and especially acid-fast bacteria.

The Polyenes

Most of the previously listed antibiotics are active primarily on bacteria and have only a limited activity on true fungi. There are, however, a number of antibiotics produced by actinomycetes that have a highly selective activity upon fungi. As pointed out previously, the first survey of the distribution of antifungal properties among actinomycetes was made by Alexopoulos. A large number of compounds have now been isolated and described under the names of actidione, fradicin, thiolutin, nystatin, candicidin, ascosin, candidin, trichomycin, antimycoin, filipin, amphotericin, and others. Most of them belong to the polyenes. Only a few of them, notably nystatin and trichomycin, have found practical application.

Recently, Ball *et al.* made a comprehensive study of the production of polyene antibiotics by species of *Streptomyces*. These polyenes were grouped together on the basis of cer-

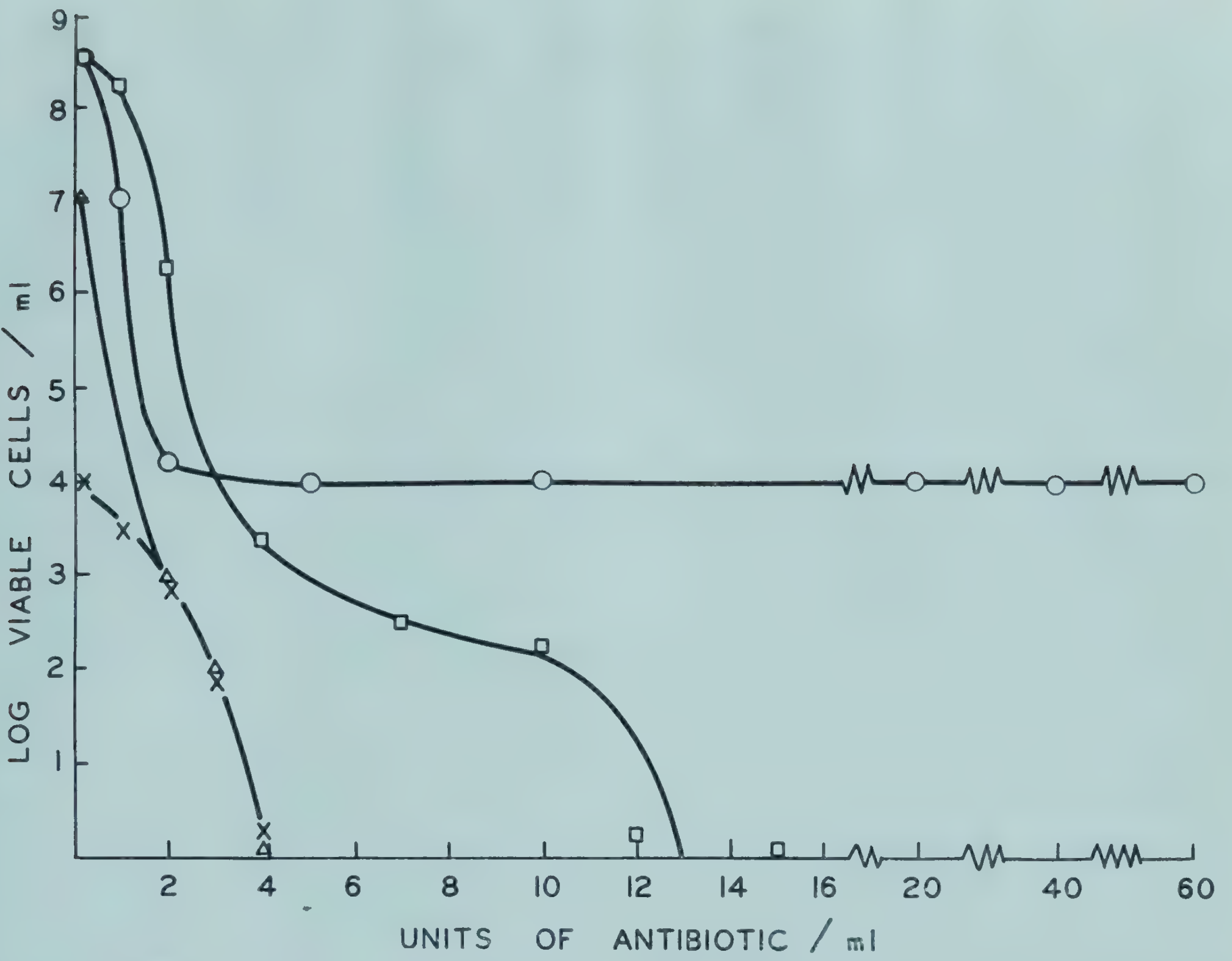


FIGURE 91. Effect of antibiotic concentration on development of resistance by *E. coli* in agar media: □, Streptomycin; ○, Grisein; △, Streptomycin + 1 unit grisein/ml; ×, Streptomycin + 5 units grisein/ml. (Reproduced from: Reynolds, D. M. and Waksman, S. A. J. Bacteriol. **55:** 749, 1948).

tain general properties that may be summarized as follows:

1. They inhibit the growth of a wide range of fungi, including yeasts, but are inactive against bacteria.
2. They show a relatively high toxicity when injected into animals, but are much less toxic when given by mouth.
3. They are of low solubility in water, dissolve more readily in aqueous solutions of the lower alcohols, and are easily soluble in aqueous pyridine.
4. They exhibit characteristic ultraviolet absorption spectra typical of polyenic chromophores (Table 66).

Vaněk *et al.* (1958) isolated from soil samples obtained from China a total of 739 actinomycetes; of these, 515 (69.7 per cent) were antibiotically active. A total of 386 cul-

TABLE 66

Classification of polyene antibiotics according to their ultraviolet absorptions (Ball *et al.*)

Group no.	Polyene type	Absorption maxima, mμ	Named antibiotics
1	Tetraene	290, 305, 318	Fungicidin, antimycoin, rimocidin, chromin, amphotericin A
2A	Pentaene	318, 333, 351	Fungichromatin, eurocidin
2B	Pentaene	325, 340, 358	Fungichromin, filipin
3	Hexaene	340, 356, 378	Mediocidin, flavacid, fradicin
4	Heptaene	360, 378, 405	Ascosin, candicidin, candidin, trichomycin, candimycin, amphotericin B, antibiotic 1968

tures were characterized by paper chromatography using agar blocks, by behavior on agar plates incorporating ion-exchange resins, and by ultraviolet absorption spectrum (to detect polyene substances). About half, or 195 cultures, produced a mixture of antibiotics of both polyene and nonpolyene type.

A detailed treatment of these and various other antibiotics produced by actinomycetes will be presented in Volume III.

Antitumor Agents

Various actinomycetes are able to exert a repressive effect against certain forms of cancer and to produce cytologically active substances. Attention has already been directed to the effect of the actinomycins, a group of antibiotics with marked cytologic properties. Azaserine is another group of compounds that are highly active against various sarcomas. A similarity in this activity and that against the yeast *Klockera brevis* was found; this made it possible to use activity against the latter in studying the purification of the antibiotic. Sarkomycin, isolated by Umezawa *et al.* in 1953, has since been studied extensively by Hooper and others. It was found to be active against experimental tumors, but did not have a great effect in human tumors. The same may be said of another antitumor agent designated as carzinophilin. A number of other substances (DON, mitomycin, sulfocidin) were found to exert marked antitumor activity, but none of these has as yet found any practical application in the control of tumors in man (Osato *et al.*).

Cavalli-Sforza *et al.* found a high frequency of antimitotic activity in the metabolic products of soil microorganisms. With the use of *Allium cepa* root tips, they observed active antimitotic strains among fungi and actinomycetes. There was no definite correlation, however, between antibiotic and antimitotic activity in the preparations thus obtained.

Antiprotozoan, Antiviral, and Antiphage Agents

Various actinomycetes are able to form substances that exert antiprotozoan effects. This is true particularly of such antibiotics as streptocin, trichomycin, and other substances active upon trichomonads; of puromycin and other agents active upon trypanosomes; and of anisomycin active upon *Endamoeba*. This field has not yet been sufficiently explored, but it has marked poten

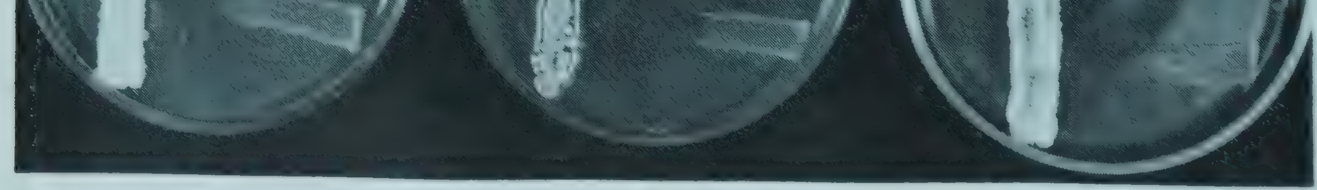


FIGURE 92. The use of bacterial strains sensitive and resistant to a given antibiotic for the identification of the particular antibiotic. *E. coli* strains (from right to left): streptomycin-resistant, streptomycin-dependent, and streptomycin-sensitive. The plates were streaked from top to bottom: original streptomycin-producing *S. griseus*; culture producing an unknown antibiotic not of the streptomycin type; an unknown streptomycin-producing culture.

tialities. Other antiprotozoan antibiotics produced by actinomycetes include congocidin, eurocidin, fermicidin, and valinomycin.

A number of antiviral substances are also produced by actinomycetes. Some of them, like the tetracyclines, are active upon the larger viruses, such as psittacosis, and have already found extensive practical application. Others are active upon the smaller viruses but are either insufficiently active or are too toxic to have found practical application. Among these, it is sufficient to mention ehrlichin, abikoviromycin, achroviromycin, noformicin, hygrosporin, and primycin.

A number of actinomycetes are also able to produce antiphage agents (Jones and Schatz). Some of these substances have been given names like chrysomycin.

Miscellaneous Antibiotics

A large number of other antibiotics produced by actinomycetes, chiefly streptomyces, have been described. Some have a wide spectrum. Others have a narrower spectrum. Still others have a very narrow spectrum. A few have found practical applications or offer promise.

Screening programs for antibiotics are being continued on a large scale. Particular attention is being directed at present to agents active against viruses and tumors. Although a number of active substances have already been isolated, none has so far given very promising practical results in the treatment of disease.

It is important to note that various antibiotics of actinomycetes are also active against plant pathogenic bacteria and fungi (Waksman *et al.*, 1944).

The great majority of antibiotics are produced by members of the genus *Streptomyces*. A few are formed by species of *Nocardia* and *Micromonospora*. Some of the thermophilic actinomycetes have been found to possess antibiotic properties (Kosmacher). None of the remaining genera (*Actinoplanes*, etc.) were

TABLE 67

Release of antibiotic activity from mycelium of S. griseus by chemical and physical treatments

(Perlman and Langlykke)

Treatment of mycelium suspension*	Antibiotic potency released by treatment, μg/ml
None	19
Heated in boiling water bath for 10 minutes	38
Exposure to sonic energy for 15 minutes	107
Addition of sufficient concentrated HCl to give:	
pH 5.1	37
pH 4.2	67
pH 3.0	109
pH 2.5	188
pH 1.8	175
Addition of sufficient 10 *N* NaOH to give:	
pH 8.0	45
pH 9.1	109
pH 9.9	161
pH 10.8	143
Addition of sodium chloride to give concentration:	
0.003 M	19
0.01 M	19
0.03 M	33
0.1 M	53
0.3 M	97
Addition of sodium sulfate to give concentration:	
0.003 M	19
0.01 M	43
0.03 M	97
0.1 M	159
0.3 M	147
Addition of sodium citrate to give concentration:	
0.003 M	25
0.01 M	64
0.03 M	142
0.1 M	129
0.3 M	130

* Samples from 4-day-old fermentation were centrifuged and the supernatant liquid assayed. The supernatant liquid contained 108 μg/ml of streptomycin. The collected solids were resuspended in distilled water to original volume, and acid, alkali, or salt was added as indicated to 10-ml aliquots. After 15 minutes of shaking on a mechanical shaker the solids were collected by centrifugation and the supernatant liquid submitted for assay.

found to exert any antagonistic effects upon other microorganisms.

Numerous other compounds were isolated from various cultures of actinomycetes. They are described in detail in Chapters 31–40 (Volume III).

The Role of Antibiotic Biosynthesis in the Metabolism of Actinomycetes

According to Perlman, the role of antibiotic biosynthesis in the metabolism of actinomycetes is of considerable significance. An analysis of the cells of *S. griseus* suggests that the amino acids do not differ in nature from those in other actinomycetes. The fact that a substantial quantity of streptomycin (usually more than half of that produced) occurs bound to the mycelium suggests that the antibiotic may form a part of the cell wall of the organism. This bound streptomycin may be released (Table 67) by treatment of the cells with acid, alkali, or ionizable salts, but not by disintegration of the cells by sonic energy, bacteriophage, or enzymatic treatment. Considerable amounts of other antibiotics, including streptothricin, the neomycins, chloramphenicol, and chlortetracycline, have been found to occur bound to the mycelium of the respective actinomycetes and may be released by treatment with acid, alkali, or ionizable salts. This binding does not appear to be a simple ion-exchange phenomenon, since addition of streptomycin to the mycelium of the organism does not result in its absorption, and the "binding" power of the mycelium is apparently not a function of its weight (Perlman and Langlykke).

Chemical Structure and Antimicrobial Activities of Actinomycete Antibiotics

The effect of chemical structure upon the biological activities of antibiotics in general and of actinomycete antibiotics in particular is discussed in detail in Chapter 31 (Volume III). It is sufficient to say here that any slight modification of the molecule may cause a profound change in the activities of the antibiotic.

As a rule, the chemical or enzymatic degradation of an antibiotic results in its loss of activity. This is not always the case, however, as shown for neamine, a degradation product of the neomycins; it retains its antibiotic properties, although the spectrum is changed. Nakamura has shown that an antibiotic of the luteomycin type ($C_{26}H_{33}NO_{12}$) gave, on acid hydrolysis, a greenish black substance, designated teomycic acid ($C_{17}H_{23}NO_{11}$), which retained its antibiotic properties. However, actinomycin treated with an enzyme preparation completely loses its activity, as shown previously.

CHAPTER 16

Decomposition of Complex Plant and Animal Residues

Actinomycetes are capable of attacking a great variety of plant and animal products, notably crop residues; they thus bring about the partial or complete decomposition of these products. Actinomycetes may, therefore, be considered, on a par with the fungi and the true bacteria, as one of the leading groups of microorganisms concerned in the destruction of organic materials and in transformation and mineralization of organic matter in nature. The literature on the soil actinomycetes, beginning with the work of Beijerinck, in the early days of the century, and continuing through recent studies, abounds in data on the abundance and activities of actinomycetes in composts and in soils rich in organic matter. The essential role of these organisms in the formation and decomposition of humus was early recognized. These processes result in bringing about the liberation of plant nutrients in available forms and are thus of great importance in plant nutrition and soil fertility.

Decomposition of Plant Materials

In a study of the decomposition of alfalfa by different groups of microorganisms, Waksman and Hutchings found that pure cultures of actinomycetes were able to decompose, in 39 to 74 days, 33 to 43 per cent of the hemicelluloses and 23.2 to 25.3 per cent of the cellulose, as well as a part of the lignin. Nearly 20 per cent of the total nitrogen in the plant residues was liberated as ammonia, thus pointing to considerable protein decomposition; much of the nitrogen must also have been used by the organisms for the synthesis of their own cell material. In the decomposition of oat straw, 24.5 per cent of the hemicelluloses were destroyed in 50 days; only little cellulose and some lignin were attacked. Cornstalks were only slightly attacked when no lime and phosphate were added but underwent rapid decomposition when these were introduced. Of the total dry plant material, the actinomycetes brought about, on an average, 20 per cent decomposition. They attacked the water-soluble substances most readily (30.5 per cent), then the hemicelluloses (16.7 per cent), and the cellulose least readily (5.4 per cent). The most striking point was the fact that the actinomycetes decomposed not only the cellulose and hemicelluloses, but the lignin in these materials as well, even to a greater extent than did the fungi, as illustrated in Table 68.

In a comparative study of the decomposition of cornstalks by several species of *Streptomyces*, alone or in the presence of a fungus or a bacterium, the streptomycetes were highly effective in decomposing a considerable amount of the cellulose and the hemicelluloses. Although their decomposing capacity was less than that of the fungus *Humicola*, especially in the absence of added

TABLE 68

Decompostion of alfalfa by pure and mixed cultures of microorganisms (Waksman and Hutchings)

Inoculum	Total residue*	Hemicelluloses	Cellulose	Lignin
Control	9.120	0.760	2.060	1.170
Soil infusion	6.529†	0.449	1.013	—
Rhizopus	8.241	0.663	2.000	1.073
Trichoderma	8.270	0.724	2.079	1.041
Trichoderma + *Streptomyces* 3065	7.983	0.649	1.962	1.023
Streptomyces 3065	7.608	0.433	1.582	0.942
Streptomyces 3065	6.570	0.508	1.538	0.906
Streptomyces + soil infusion	5.064	0.384	0.925	0.821

* Values are given in grams on basis of dry material.

† The amount of the constituents decomposed by each organism or combination of organisms can easily be calculated by subtracting the material left from that of the control.

lime, their activities were highly significant, and particularly when one remembers that the fungus used in this experiment was one of the most effective in the decomposition of plant materials.

A number of factors, such as reaction, aeration, moisture, and temperature, exert a considerable influence on the decomposition of plant materials by actinomycetes. This is illustrated in the effect of added lime upon their activities, as shown in Table 69. The maximum decomposition of dried blood by actinomycetes, as measured by ammonia formation, was found to take place at pH 5.8 to 7.7; some organisms showed some activity at pH 5.0, but very little decomposition of this material took place at pH 4.0 and at pH 8.8.

Decomposition of Cellulose and Hemicelluloses

As has been pointed out, various actinomycetes are capable of decomposing different hemicelluloses. Waksman and Diehm made an extensive study (Tables 70–72) of the decomposition by actinomycetes of a variety of hemicelluloses in sand, soil, and liquid media. They found them capable of bringing about considerable decomposition of these carbohydrates both in an isolated and chemically

TABLE 69

Influence of lime and associative organisms upon the growth of actinomycetes on cornstalks (Waksman and Hutchings)

Decomposition in per cent

Organism	Treatment with $CaCO_3$	Total decomposed	Water-soluble organic matter	Hemicellulose	Cellulose
Control	−	0	0	0	0
Streptomyces 3065	−	1.0	4.6	0	2.7
Humicola	−	26.3	42.0	4	3.8
Humicola + *Streptomyces* 3065	−	31.1	37.9	21.1	7.1
Streptomyces 3065	+	21.6	28.5	19.3	6.6
Streptomyces 3018	+	23.7	29.7	16.5	9.6
Streptomyces 3310	+	14.7	33.4	14.3	0
Streptomyces 3065 + *Ps. fluorescens*	+	24.2	35.3	22.6	7.1
Humicola	+	25.1	43.5	9	12.0
Humicola + *Streptomyces* 3065		24.3	41.7	18.5	6.4

TABLE 70

Decomposition of different polysaccharides by various streptomyces (Waksman and Diehm)

Milligrams per flask of sand medium

Streptomyces No.	Mannan		Xylan		Galactan	
	Found	Decomposed	Found	Decomposed	Found	Decomposed
Control	305.5	—	152.0	—	170.2	—
26	31.3	274.2	38.7	133.3	119.7	50.5
40	15.1	290.4	25.2	126.8	97.2	73.0
48	25.9	279.6	29.7	122.3	—	—
50	17.8	287.7	—	—	69.3	100.9
53	23.9	281.6	—	—	99.0	71.2

purified state and in a natural condition in the plant materials. Mannans and xylans were attacked particularly. Decomposition of laminarin by actinomycetes was studied by Chesters *et al.* (1955). The formation of the enzyme xylanase by actinomycetes was shown in Chapter 11. Numerous other investigators have demonstrated the ability of actinomycetes to decompose cellulose and various hemicelluloses. In the degradation of cellulose in the intestinal canal, certain actinomycetes probably play a part, as shown by Hungate (1946) for a species of *Micromonospora.* The active part played by actinomycetes in cellulose decomposition under high salt concentration, with the result that black muds are formed, has been established by Rubentschik (1928, 1932).

Cellulose decomposition in composts, under thermophilic conditions, was first demonstrated by Tsiklinsky (1899) and by Schütze (1908); later, extensive studies were made by Waksman and Cordon (1939), Waksman *et al.* (1939), Waksman and Corke, and more recently by Henssen. Other studies on cellulose decomposition by actinomycetes were made by Bokor (1930), Meyer (1934), and others.

TABLE 71

Comparative decomposition of xylan in corncobs by fungi and actinomycetes (Waksman and Diehm)

Milligrams per flask

Organism	Found	Decomposed
Control	405.5	—
Rhizopus	187.9	237.6
Penicillium	223.6	181.9
Trichoderma	331.0	74.5
Asp. niger	302.9	102.6
Streptomyces 26	372.1	33.4
Streptomyces 50	362.9	42.6
Streptomyces 40	306.2	98.3

TABLE 72

Relative decomposition of galactan in Irish moss by different microorganisms (Waksman and Diehm)

Milligrams per flask

Organism	Found	Decomposed
Control	382.3	—
Rhizopus	259.7	122.6
Penicillium	263.0	119.3
Trichoderma	265.7	116.6
Asp. niger	281.3	101.0
Streptomyces 26	263.0	119.3
Streptomyces 33	268.9	113.4
Streptomyces 35	287.3	95.0
Streptomyces 40	253.8	128.5
Streptomyces 50	241.9	140.4

Decomposition of Proteins

The ability of actinomycetes to take an active part in protein decomposition is also highly significant. In a study of the effect of dried blood *versus* rye straw upon the development of fungi and actinomycetes in differently treated soils (Tables 73 and 74), it was found that the addition of protein-rich materials greatly stimulates the development of actinomycetes as compared to other groups of microorganisms.

The ability of actinomycetes to decompose proteins into amino acids and ammonia was first shown by Macé. In view of the fact that actinomycetes synthesize considerably less mycelium than do fungi, only small quantities of nitrogen are assimilated into complex cell material. Most of it is liberated free in the form of ammonia. Although actinomycetes also utilize nonnitrogenous organic materials for cell synthesis, such materials do not exert such a depressing effect upon the liberation of ammonia as do bacteria and fungi.

Nicolaieva, in a study of protein decomposition by eight cultures of actinomycetes, found that the proteins were completely degraded. She came to the conclusion that actinomycetes take an active part in soil processes, leading to the mineralization of soil organic matter. As shown previously (Chapter 7), Waksman and Starkey demonstrated

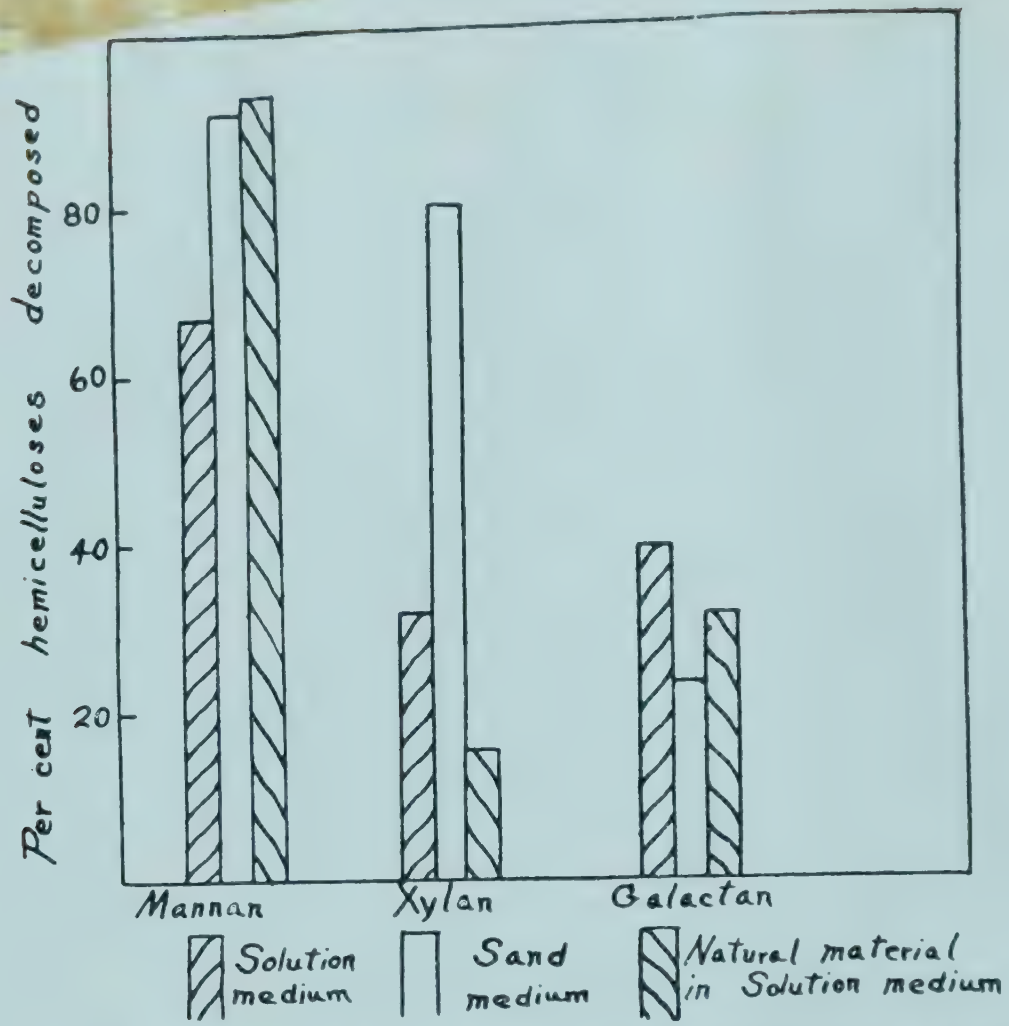


FIGURE 93. Rate of decomposition of various hemicelluloses by actinomycetes in different media, in 6 weeks (Reproduced from: Waksman, S. A. and Diehm, R. A. Soil Sci. 32: 115, 1931).

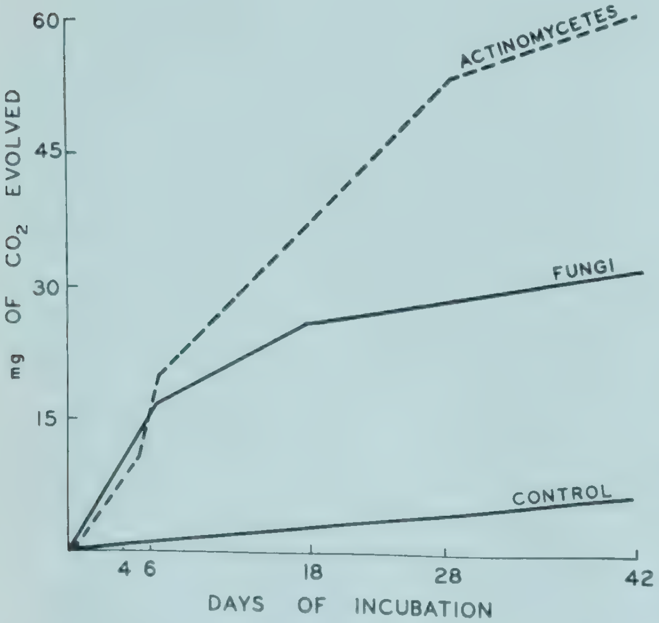


FIGURE 94. Decomposition of xylan (Reproduced from: Waksman, S. A. and Diehm, R. A. Soil Sci., 32: 113, 1931).

TABLE 73

Influence of rye straw (1 per cent) upon the development of fungi and actinomycetes in various soils after 10 days (Waksman and Starkey)

Annual soil treatment	pH	Fungi		Actinomycetes, thousands	
		Start	End	Start	End
Manure, minerals	5.5	87,300	750,000	1,800	2,800
Manure, lime, minerals	6.7	19,700	24,000	3,360	2,800
Untreated	5.1	115,700	600,000	1,260	200
Lime alone	6.5	20,000	19,000	2,760	1,900
$NaNO_3$, minerals	5.8	73,300	650,000	1,500	1,800
$(NH_4)_2SO_4$, minerals, lime	6.0	25,700	47,000	2,700	1,800

that actinomycetes actively decompose plant proteins, liberating the nitrogen as ammonia. Decomposition of protein fibers by actinomycetes has been studied by Goldsmith. The enzymatic mechanisms involved in protein decomposition by actinomycetes are discussed in Chapter 11.

Lignin Decomposition

In connection with the decomposition of plant materials in soils and in composts the effect of actinomycetes on the lignin is of particular interest. It is now well recognized that the lignins and the proteins contribute greatly to the formation of humus in soils and in composts. As the plant materials are decomposed by fungi and bacteria, there is usually an increase in the concentration of the lignin, since most of these organisms do not attack this complex very readily (Waksman). This accumulation of the lignin is paralleled by an increase in ash content and often in the protein content in the case of nitrogen-poor materials, and by a decrease in the total dry material. Through their ability to attack the resistant lignins, the actinomycetes have the capacity to leave an organic residue with a lower lignin content.

Decompostion of Other Organic Complexes

Actinomycetes are capable of growing on and decomposing a great variety of other organic materials. These include paraffins (Baldacci, 1947), waxes, rubber, and building materials (McLachlan, 1946). The ability to attack paraffins is characteristic of certain nocardias, as shown elsewhere in the description of the individual species in Chapter 23 (Volume II).

Formation and Decomposition of Humus

On the basis of the foregoing observations, the conclusion may easily be reached that actinomycetes take an active part in the formation and decomposition of organic matter or humus in the soil. This comprises both nitrogenous and nonnitrogenous organic substances. Because of their ability to attack native lignin, actinomycetes may even be expected to play a unique role in the formation and transformation of humus materials.

TABLE 74

Influence of dried blood (1 per cent) upon the numbers of fungi and actinomycetes in various soils after 12 days (Waksman and Starkey)

Annual soil treatment	pH	Fungi		Actinomycetes, thousands	
		Start	End	Start	End
Manure, minerals	5.5	87,300	2,079,950	1,800	190,900
Manure, lime, minerals	6.7	19,700	73,300	3,360	6,000
Untreated	5.1	115,700	1,438,300	1,260	2,200
Lime alone	6.5	20,000	125,000	2,760	500
$NaNO_3$, minerals	5.8	73,300	1,871,650	1,500	128,700
$(NH_4)_2SO_4$, minerals, lime	6.0	25,700	311,600	2,700	42,700

The role of actinomycetes in the formation of dark colored compounds and the possible bearing of these compounds upon humus formation were first pointed out by Beijerinck. He emphasized that the black pigment produced by some of these organisms on protein media may function as an oxidizing agent. On the basis of this, he tried to postulate their significance in natural processes, notably in the humification of soil organic matter. He correlated this with the abundance of actinomycetes at different soil depths.

The results of Beijerinck were confirmed and further extended by Fousek, Münter, Krainsky, and Waksman, and later by von Plotho, Lantsch *et al.*, and Scheffer *et al.* The ability of various streptomyces to give rise to dark brown substances, comparable to humic acids, was demonstrated recently by Flaig *et al.* (1952), Küster (1952), and Beutelspacher (1952). The formation of such substances depends on the nitrogen source and on the nature of the organism. On prolonged incubation of the cultures and proper chemical manipulations, preparations were obtained that showed great similarity to the humic acids occurring in natural soils. Certain amino acids can also give rise to brown substances as a result of the growth of some actinomycetes.

Pure cultures of an organism belonging to the genus *Streptomyces* and of the fungus *Trichoderma* were found by Waksman to decompose more peat material than did a complex soil microbiological population (Table 75). The amount of decomposition was measured by the amount of CO_2 and ammonia formed. The ratio of the carbon decomposed to that of the nitrogen liberated was lower for the pure cultures than for the complex population. This indicated that the pure cultures attacked more of the nitrogenous constituents than did the total soil population.

Actinomycetes are thus shown to be capable of decomposing resistant humus materials in the soil and bringing about the liberation of the constituent elements essential for plant growth. The nitrogen stored up in the humus is changed to ammonia, which is later oxidized to nitrate. Liming of soil and draining of swampy areas favor the development of actinomycetes as well as the decomposition of the soil organic matter. This process is of considerable importance to soil fertility. According to Fousek, an increase in plant growth is obtained by inoculating actinomycetes into the soil, thereby bringing about increased decomposition of the organic matter. This observation has not been fully confirmed as yet.

Table 75

Decomposition of sedge and reed peat by microorganisms (Waksman and Stevens)

On the basis of 20 gm of dry peat decomposed for 28 days under favorable moisture conditions

Organism	CO_2 formed, mg C	Nitrogen as ammonia and nitrate, mg N	Ratio of liberated C:N
Streptomyces	87.7	13.4	6.5
Trichoderma	88.4	14.1	6.1
Soil infusion	68.7	9.6	7.2

Conn also emphasized the importance of actinomycetes in the decomposition of organic residues in the soil. Colonies of these organisms, mostly streptomycetes, were found developing on plates seeded with soil infusions. They made up 20 per cent of the total number of organisms in cultivated soils and 37.5 per cent of organisms from sod soils. The longer the time during which grass was grown in the soil, the larger was the proportion of the actinomycetes to the total population developing on the plate. A soil containing 2,900,000 actinomycetes per gram, when treated with grass roots, gave an increase in numbers to 6,000,000 in 2 weeks. Both dead grass roots mixed with the soil and grass growing in the soil were found to have a marked stimulating effect upon the development of these organisms.

Decomposition of High-temperature Composts

In the decomposition of plant materials, especially in composts of stable manures and artificial composts, high temperature or thermophilic conditions are attained. Under these conditions, actinomycetes play an eminent part in the decomposition process, as brought out first by Globig, Miehe, and others. It was later studied extensively by Waksman, Umbreit, and Cordon, who reported that at temperatures of 50 to 65°C, the actinomycetes were highly active in the decomposition processes. Soils receiving stable manure contained an abundant population of actinomycetes, notably thermophilic forms, as illustrated in Figure 96. Waksman and Hutchings found that actinomycetes may be more active in the breakdown of plant constituents in mixed populations than in pure culture. A similar

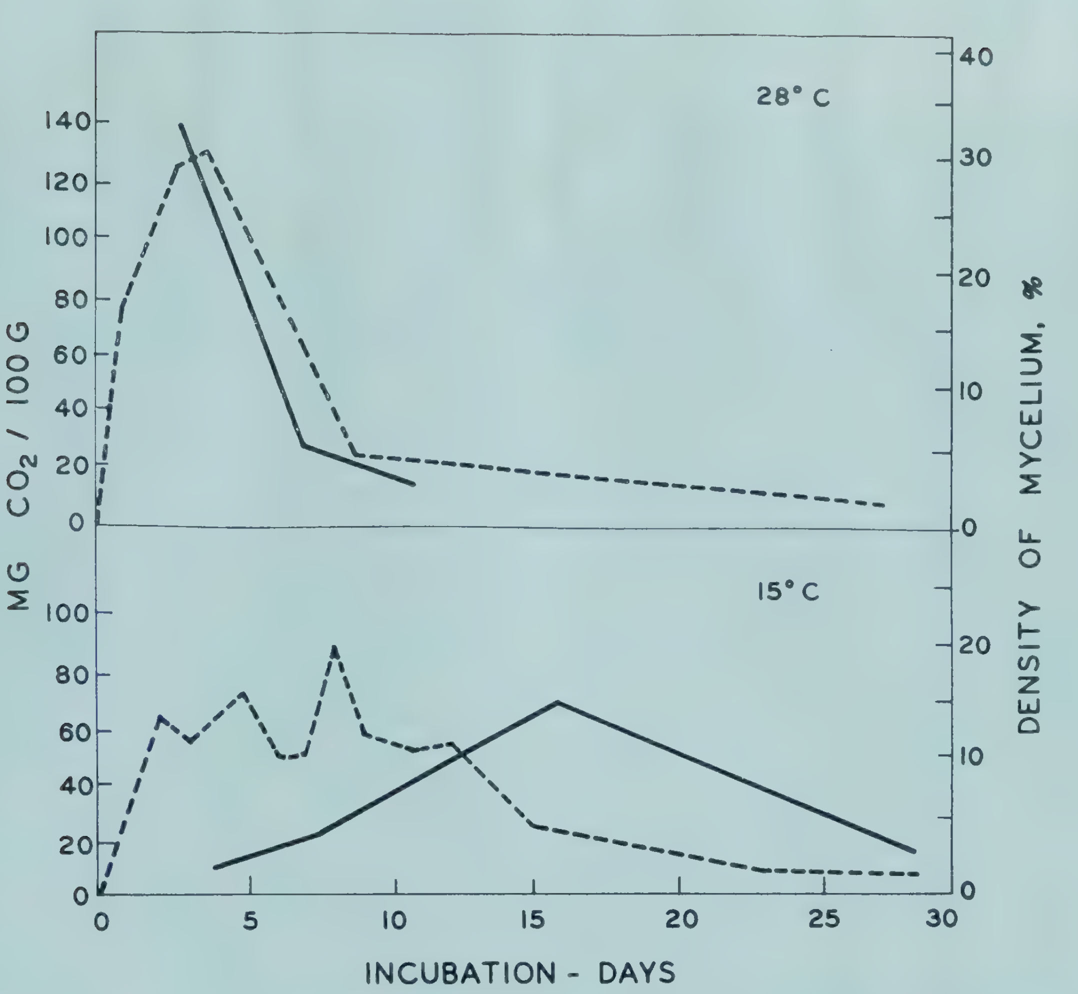


FIGURE 95. Influence of temperature upon growth and CO_2 production by actinomycetes. Continuous line: density of mycelium. Broken line: CO_2 evolved (Reproduced from: Jensen, H. L. Proc. Linnean Soc. N.S. Wales **68:** 70, 1943).

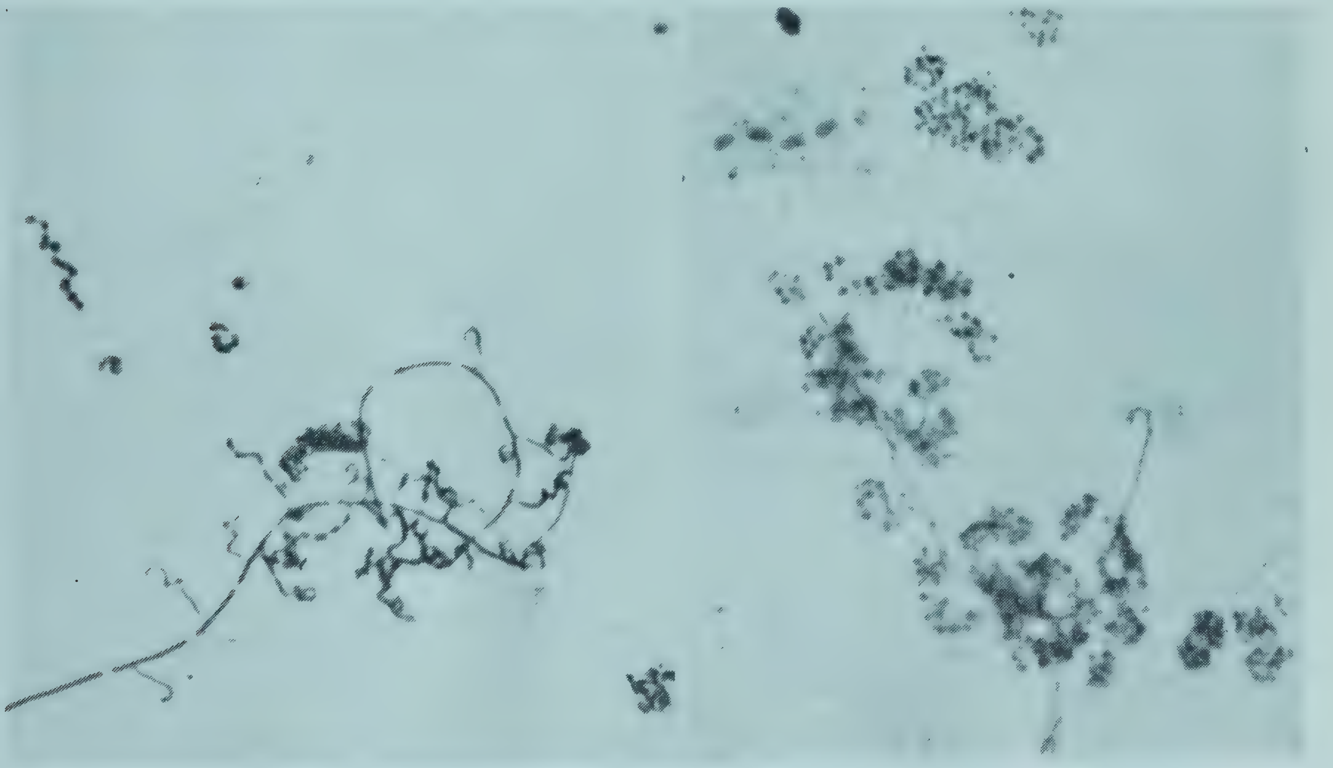


FIGURE 96. Typical growth of actinomycetes in high temperature composts, as illustrated by contact slide method.

population picture was obtained by Kaila. Henssen recognized that certain species of streptomyces and nocardias are found abundantly in thermophilic composts. He emphasized, however, the abundance of specific thermophilic groups of actinomycetes, such as *Thermoactinomyces* and certain newly created thermophilic genera, as shown in Chapter 29 (Volume II).

Actinomycetes as Agents of Deterioration and Spoilage

Through their ability to attack resistant compounds and through their universal occurrence, actinomycetes may frequently be responsible for considerable damage to foodstuffs and textiles. As a rule, actinomycetes are usually not considered important agents of deterioration and spoilage. It can easily be established, however, that, under certain special conditions, actinomycetes may play a far more important role in these processes than is commonly supposed. It is sufficient to present the following evidence:

1. Certain foodstuffs are known to deteriorate as a result of characteristic earthy and pungent flavors and odors imparted by actinomycetes, as pointed out previously. This is true of milk, cacao, potable waters, and fish. The flesh of fish is tainted through absorption from the water of the odoriferous substance produced by actinomycetes. Cacao can be damaged in a similar manner. The damage to Brazil nuts by actinomycetes has been suggested; an organism, described as *A. brasiliensis*, a streptomyces, was isolated by Spencer from the shells of such nuts.

2. Certain fabrics, notably woolens, cotton goods, and paper, may be stained or actually destroyed by actinomycetes. Although the rate and extent of such destruction cannot be compared with those caused by fungi or certain bacteria, especially under humid and high temperature conditions, the actinomycetes produce a variety of stains (yellow, pink, red, black) on cloth and on paper, especially in books, and thus cause considerable damage.

3. Bredemann and Werner isolated from soils a chromogenic actinomycete capable of actively decomposing salts of butyric acid. The culture withstood heating for 5 minutes at 80°C. The illustrations given in this report suggest that the organism was a *Micromonospora*. The culture was warty and brown in color. It produced a soluble rose pigment.

4. As a result of extensive studies carried on in connection with the deterioration program during the Second World War in the Pacific, various cultures of actinomycetes were isolated. Their exact part in causing deterioration of service materials has not been fully established.

References to numerous other forms of potential deterioration of essential materials by actinomycetes are found in the literature. Galli-Vallerio and Reiss pointed out the ability of actinomycetes belonging to the streptomyces to attack photographic paper, both developed and undeveloped. They found such cultures in the wash water used in photographic work. The ability of actinomycetes to attack rubber, paraffin, and other complex materials has already been mentioned. The nature of the damage that may thus be caused has not been determined.

CHAPTER 17

Causation of Animal Diseases

Saprophytism and Parasitism

The substrate on which microbes normally live has frequently been used as a basis for classification and differentiation of these organisms. The normal existence of an organism on dead organic and inorganic residues has come to indicate its saprophytic nature. Parasitism has come to indicate the normal existence of an organism on living bodies of higher plants and animals and of microorganisms. On the basis of their ability to live exclusively or electively on living substrates, some of the parasites are classed either as obligate or as facultative.

A parasite may also be virulent, if it has the capacity to infect a living organism. Virulence varies greatly in nature and intensity, depending not only upon the species of the infecting organism, but also upon the strain and its previous history, as well as upon the nature of the host. The mode of infection, the ability of the parasite to spread through the various tissues of the host, its toxic manifestations, the degree of communicability, all contribute to the intensity of virulence.

An organism may be made to increase or decrease its virulence by serial animal passage or by growth of the culture under saprophytic conditions. Phenomena of dissociation in the culture and the development of resistance to a particular treatment also contribute to the degree of its virulence. Often such changes are accompanied by a change n the morphology of the organism or in its mmunological properties.

The above considerations have a particular application to the analysis of the pathogenic properties of actinomycetes. The most important and most highly significant comment to be made in this connection is that although actinomycetes are abundant and widely distributed in nature, they are able to cause only very few human and animal diseases. On the contrary, many actinomycetes are able to produce antibiotic substances which have found extensive application in the treatment of such diseases, especially those caused by bacteria.

Actinomycetes as Causative Agents of Disease

Although actinomycetes have been isolated from various organs and excretions of diseased human and animal bodies, they have not always been the causes of the diseases. At present, there are two major diseases with which actinomycetes are usually associated. One is caused by anaerobic organisms and is known as actinomycosis. The other is caused by aerobic organisms and is known as nocardiosis. Various other synonyms, such as streptothricosis and maduramycosis were once used to designate these or similar diseases, but these terms were gradually discarded (Foulerton, 1910).

Cope insisted upon adopting the generic name *Actinomyces* for the "whole group of organisms" and actinomycosis for the disease caused by them, since the name "is

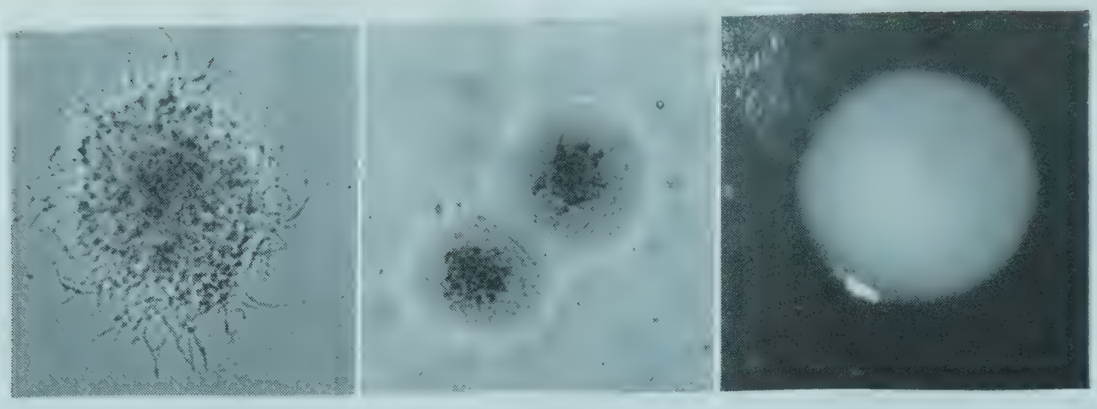


FIGURE 97: *A. bovis.* (a) 48 hour, ×300; (b) ×100, (c) 6 to 7 days, ×20 (Reproduced from: Thompson, L. Proc. Staff Meet. Mayo Clinic **25:** 84, 1950.)

graphic in character, descriptive in nature, and sanctioned by long usage."

As reviewed previously (Chapters 1 and 4) the "lumpy jaw" disease of cattle was recognized for many years prior to modern developments of microbiology. A similar, if not the same, disease was also known to occur in man. In 1876, Bollinger observed that a branched organism was constantly associated with the diseased jaw of a cow. He recognized the organism as the cause of the disease, and placed this material in the hands of the botanist Harz. The latter examined the granules, observed the characteristic radiation of the organism, and named it *Actinomyces bovis* and the disease "actinomycosis." As pointed out previously, Harz never isolated this organism in culture.

A comprehensive review of the human and animal diseases caused by actinomycetes is found in the work of Pinoy (1913), Poncet and Berard (1928), Cope, Dodge, Bullock, Conant (1944), Topley and Wilson (1946), Emmons, Gonzalez-Ochoa, Mariat, and numerous others.

Isolation of Specific Actinomycetes from Human and Animal Diseases

Simultaneously with the work of Bollinger and Harz, J. Israel (1878) was studying pathological material from pyemia and suppuration in the neck of man; he observed granules which contained mycelium similar to that described by Bollinger in cattle. The presence of a staphylococcal infection prevented him from establishing definitely that an actinomyces was the causative agent of human actinomycosis. Ponfick (1879) is usually credited with having reported the first accurate observation of the human infection.

Johne (1882) took serious exceptions to Ponfick's claims, however. He stated that Langenbeck was the first to observe, in 1845, the occurrence of actinomycetes in the human body, but he emphasized that it was Israel (1878), in spite of Ponfick's claims, who first recognized and described an actinomyces as a causative agent of human diseases. In the case of animals, Hahn (1870) was said by Johne to have observed such an organism on cattle tongue, but it was Bollinger (1876) who first recognized and described its infectious nature. Johne gives Ponfick only the credit for recognizing the identity of the human and animal pathogens.

In 1885, Israel published the results of a study of actinomycosis based upon 38 cases; he thereby definitely elucidated the clinical aspect of the disease. In 1885, Bostroem claimed to have succeeded in isolating pure cultures of the organism from cattle; later, in 1890, he claimed further to have isolated such cultures also from human lesions. Since these cultures were aerobes, it is now generally assumed that he isolated air contaminants. Bostroem's identification proved to be incorrect and highly misleading. In 1889, Bujwid obtained from human actinomycosis pure cultures of the anaerobic organism, the true causative agent of the disease; its growth on glycerol agar resembled that of the tubercle bacillus.

In 1891, Wolff and Israel reported on a comprehensive investigation of the morphology and pathogenicity of the organism causing actinomycosis. They considered the microaerophilic actinomyces to be the only causative agent of the human and bovine forms of the disease. It may be of interest to quote here the results on the cultivation of the organism as reported by them (Tr. by Wright):

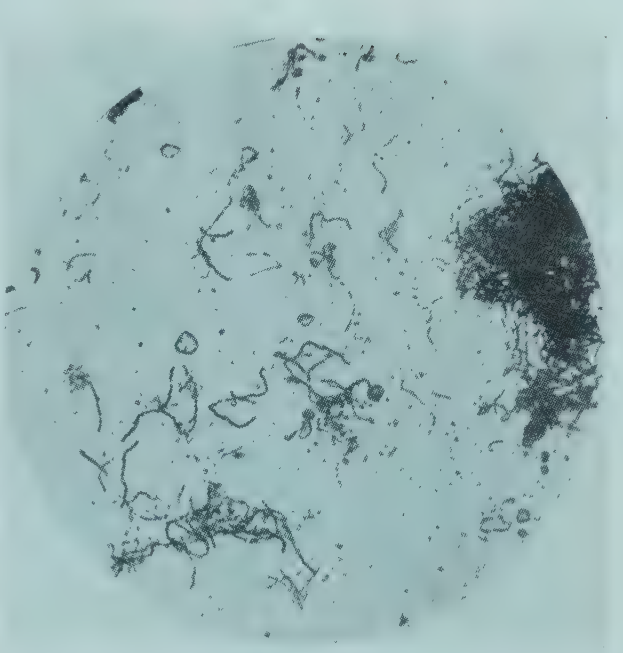


FIGURE 98. Stained dental scum (Reproduced from: Náeslund, C. Acta Pathol. Microbiol. Scand. 2: 140, 1925).

> "It grew best under anaerobic conditions and did not grow at room temperature. On the surface of anaerobic agar slant cultures on the third, fourth and fifth day numerous minute isolated dew-drop-like colonies appeared, the largest pin head in size. These gradually became larger and formed ball-like, irregularly rounded elevated nodules varying in size up to that of a millet seed, exceptionally attaining the size of a lentil or larger. As a rule the colonies did not become confluent, and an apparently homogeneous layer of growth was seen to be made up of separate nodules if examined with a lens. In some instances the colonies presented a prominent center with a lobulated margin and appeared as rosettes. A characteristic of the colonies was that they sent into the agar root-like projections. In aerobic agar slant cultures no growth or a slow and very feeble growth was obtained. In stab cultures the growth was sometimes limited to the lower portion of the line of inoculation or was more vigorous there. In bouillon, after three to five days, growth appeared as small white flakes, partly floating and partly collected at the bottom of the tube. Growth occurred in bouillon under aerobic conditions, but was better under anaerobic conditions. The microorganism in smear preparations from agar cultures appeared chiefly as short homogeneous, usually straight, but also comma-like or bowed rods, whose length and breadth varied. In many cultures short-clump rods predominated, and in others longer, thicker, or thinner individuals were more numerous. The ends of the rods often showed olive or ball-like swellings. In egg cultures growth occurred in the form of white opaque granules, the largest about the size of a pin head. Microscopically the growth was characterized by the development of long filamentous forms forming a network. The longer filaments were arranged more or less radially, were straight or wavy or spiral and sometimes branched. Cultures on potato or in milk are not mentioned. Some twenty guinea-pigs and rabbits were inoculated, most of them in the peritoneal cavity with pieces of agar culture. Eighteen animals were killed after four to seventeen weeks, and four were still alive seven to nine months after the inoculation. Seventeen rabbits and one guinea-pig showed at the autopsy tumor growths mostly in the peritoneal cavity and in one instance in the spleen. In the four animals still living tumors were to be felt in the abdominal wall. The tumors in the peritoneal cavity were millet seed to plum size, and were situated partly on the abdominal wall and partly on the intestines, the omentum, and mesentery, and in the liver or in adhesions. Frequently small millet-seed-sized tumors were situated in the neighborhood of the larger tumors. While the surface of the small tumors was always smooth, the surface of the larger tumors showed small hemispherical prominences, giving them the appearance of conglomerates of smaller tumors. On section the larger tumors presented a tough capsule from which anastomosing septa extended inward enclosing cheesy masses. Microscopical examination of the tumors showed in all cases but one the presence of typical actinomyces colonies, in most cases with typical clubs. The general histological appearance of the tumors was like that of actinomycotic tissue."

In 1896, Kruse named this organism *Streptothrix israeli*. Wright, in 1905, suggested changing the name to *Actinomyces bovis*, since he considered this organism to be identical with that recorded by Harz. It is of interest to quote from Wright:

> "Branching filamentous micro-organisms have been isolated in pure culture from thirteen cases of actinomycosis in man and two cases in cattle. The micro-organism seems to be all of one species—grows well only in agar and bouillon cultures and in the incubator; in the other usual culture media and at room temperature, it grows only very little or not at all. It is essentially an anaerobe. It does not form spore-like reproductive elements."

Wright added:

> "In cultures its colonies are similar in character to colonies of the microorganism in the lesions of actinomycosis. If colonies of the microorganism are immersed in animal fluids, such as blood serum and serous pleuritic fluid, the filaments of the colonies in immediate contact with the fluid may, under certain unknown conditions, become invested with a layer of hyaline eosin-staining material of varying thickness, and the filament may then disappear. Thus structures are produced that seem to be identical with the characteristic 'clubs' of actinomyces colonies in the lesions."

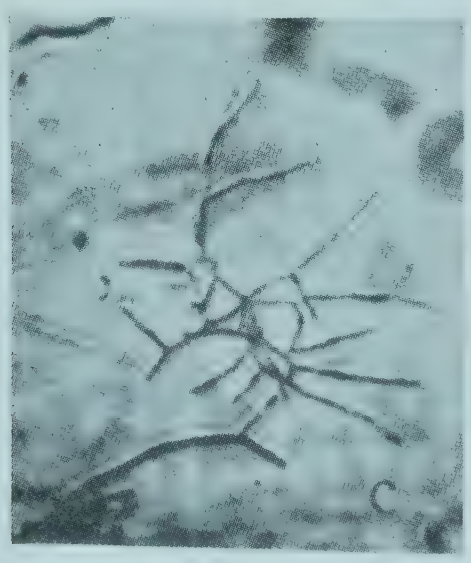


FIGURE 99. *N. asteroides* in sputum (Reproduced from: Kirby, W. M. M. and McNaught, J. B. Arch. Internal Med. 78: 8, 1946).

Wright emphasized further:

> "Between *Actinomyces* from the human and bovine cases I have found no difference which seems to me to be sufficient to justify their classification as separate species.
>
> "I do not accept the prevalent belief, based on the work of Bostroem, Gasperini, and others, that the specific infectious agent of actinomycosis is to be found among certain branching microorganisms, widely disseminated in the outer world, which differ profoundly from *Actinomyces bovis* in having spore-like reproductive elements. I think that these should be grouped together as a separate genus with the name of *Nocardia*, and that those cases of undoubted infection by them should be called nocardiosis and not actinomycosis. The term actinomycosis should be used only for those inflammatory processes the lesions of which contain the characteristic granules or 'drusen.'
>
> "Because the microorganism here described does not grow well on all the ordinary culture media and practically not at all at room temperature, I did not believe that it has its usual habitat outside of the body. It seems to me very probable that *Actinomyces bovis* is a normal inhabitant of the secretions of the buccal cavity and of the gastro-intestinal tract, both of man and animals, but I have no proof of this to offer at the present time. In these secretions it should not exist in the characteristic forms seen in the lesions, but it probably will be found in the form of fragmented filaments growing in company with bacteria, and not now differentiated from them. I believe that the part played by foreign bodies so frequently found in actinomycotic lesions is not that of the carrier of the microorganism into the tissues from without, but that the foreign body, by its traumatic and irritative effect furnishes a nidus in the tissues for *Actinomyces* which enters therein with the secretions from the buccal cavity and gastro-intestinal tract, develops into characteristic colonies, and produces lesions which we call actinomycosis."

Colebrook (1920) considered as actinomycosis only those cases that showed suppurative lesions, the pus of which contained "granules" visible to the naked eye and composed of a framework of a filamentous organism. He did not follow Wright in considering the presence of "clubs" ("ray-formation") at the periphery of the granules as an absolute requirement in the diagnosis of actinomycosis. In isolating pure cultures, Colebrook used the method of Gordon, which consisted of implanting a granule into a tube of blood-broth under an oil seal. Growth of the organisms was always slow, the primary culture requiring 3 to 8 days and subcultures 2 to 6 days. Growth never occurred at 22°C. All strains isolated showed preference for anaerobic growth, but several were capable of aerobic growth after some subculturing. Occasionally, even primary aerobic cultures were obtained after 10 to 14 days' incubation. The organism isolated from 21 cases was definitely of the *A. bovis* type. It showed coarse agglutination with the serum of heavily infected patients, as well as with the serum of infected rabbits. Colebrook dismissed the idea that infection with such a fragile organism and of such slight viability could occur from outside "natural" sources, as claimed by Bostroem and others. He considered the organism as a common inhabitant of the human alimentary tract.

Lieske tried to justify the apparently conflicting observations on the aerobic and anaerobic organisms by emphasizing the fact that the anaerobic forms tend to become aerobic after several subcultures. This led him to suggest that one type might be converted into the other.

Naeslund (1931) finally established the fact that the great majority of actinomycosis cases are caused by a preferentially anaerobic organism. Certain cases, however, affecting the lung and skin may be caused by aerobes, which come from the inhalation of dust containing the organism (Biggart).

Cope suggested recognition, for clinical purposes, of two main groups of actinomycetes:

1. A very large group including those forms which grow in the natural state in soil and on organic residues. These are hardy organisms growing easily and quickly at room temperature on all ordinary media in the ordinary atmosphere. They are then aerobic in nature. Very few of them are pathogenic.

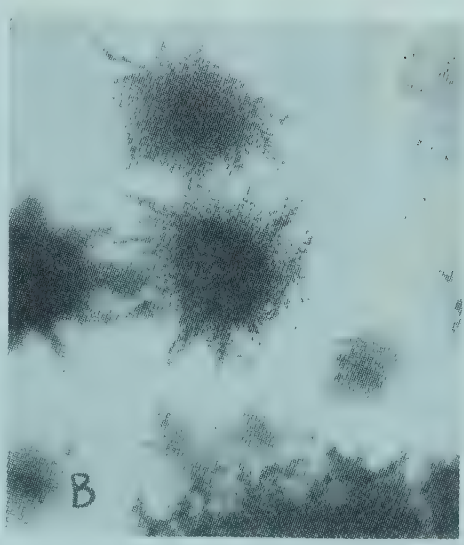


FIGURE 100. *N. asteroides* on nutrient agar (Reproduced from: Kirby, W. M. M. and McNaught, J. B. Arch. Internal Med. 78: 8, 1946).

2. Those that are preferentially anaerobic. They comprise a much smaller group. Clinically they are more important. They are much more delicate and more difficult to grow than the aerobic type. They grow best in the absence of oxygen or with only a limited supply. They are not found outside the body, and are responsible for nine-tenths of the cases of actinomycosis in man.

The first group comprises those organisms that are now recognized as members of the genera *Nocardia* and *Streptomyces*. Numerous reports are found in the literature concerning the isolation, from human or animal blood or pus, of actinomycetes belonging to this group. Many investigators were inclined to consider them largely as dust contaminants. Some (Thjotta and Gundersen) looked upon them not as etiologic agents of particular diseases but as saprophytes found in the respiratory tract (in the throat and on the tonsils) and gaining entrance into the blood of the patient when the body defenses were low.

There were numerous other reports of the occurrence of streptomyces in human organs, as in the ear (Odom *et al.*), in tumors (Leyton and Leyton), and in tear ducts and glands (Gruter). Mackinnon and Artagaveytia-Allende (1956) examined 38 strains of aerobic actinomycetes producing localized mycetoma in various zones of the world. They identified these organisms as species of *Streptomyces* and *Nocardia*.

The isolation of *Micromonospora* cultures from animal and human cases has also been reported. Morquer and Comby found (1943) *M. caballi* to be parasitic on the horse, but not on rabbits, rats, or mice.

Acton and McGuire observed the occurrence in India of a red actinomycete that produces keratolytic changes in the skin of the hands and feet, causing lesions known as keratolysis plantare sucatum, mango toe, cracked heel, paronychia, onychomycosis, and vesicular eruptions. It is commonly believed that these lesions are caused by walking barefooted on damp soil, particularly soil contaminated by horse manure. The organism was actually recovered from both horse and cow manure; it had a marked lytic action on the horny layer of the epidermis of the soles of the feet and sometimes on the palms of the hand. In culture, the colonies were red or black, with deep mycelium penetrating the media. On microscopic examination the organism showed fine hyphae, about 0.8 μ in diameter. The conidia were small and round, and formed along the course of the aerial hyphae, at the ends or growing out laterally. At first they were single, but in old cultures they were grouped and surrounded the aerial hyphae. The name *A. keratolytica* was proposed, but a study of the illustrations shows the organism to be a *Micromonospora*. The organism causing lumpiness of matted wool in sheep is definitely a *Nocardia* and not an *Actinomyces*, as claimed by Bull.

Heymer (1957) found a *Streptomyces* (*S. coelicolor*) in relatively great abundance in the microflora of human beings, notably in the sputum, tonsilar crypts, and skin. This organism exerted an antagonistic effect upon various human pathogenic fungi and yeasts. It was suggested that such organisms may play an important role in keeping the microbiological equilibrium in the human body.

Further information, some of which is highly fragmentary, on the causation of different animal diseases by actinomycetes is found elsewhere in this treatise, notably in Chapters 24, 25, and 30, Volume II. In other cases, where some degree of certainty exists of the disease causation, the organisms have not been sufficiently studied to enable ascertainment of their exact relationship to others now well recognized. The introduction of new systems of classification has complicated further the recognition of some of the disease-producing forms. It was simple enough when the organisms could be classified under "Actinomyces" or "Streptothrix." It was still relatively simple to place the anaerobes under *Actinomyces* and the aerobes under *Nocardia.* With the introduction of the newer genera, notably *Streptomyces* and *Micromonospora,* it became very difficult to decide when an aerobic organism should be placed in the *Streptomyces* or in *Nocardia.* In the excellent work of Erikson, for example, the accurate descriptions permit one to decide where certain cultures might preferably be placed. Other, more recent investigators were so uncertain of the systematic position of the particular cultures as to designate them by two generic names, such as *Nocardia* (*Streptomyces*). Still others (Gordon) were not averse to lumping many cultures, showing only minor variations from one another, under a particular species.

The present discussion may be limited, however, to two types of disease caused by actinomycetes and most frequently designated as actinomycosis and nocardiosis.

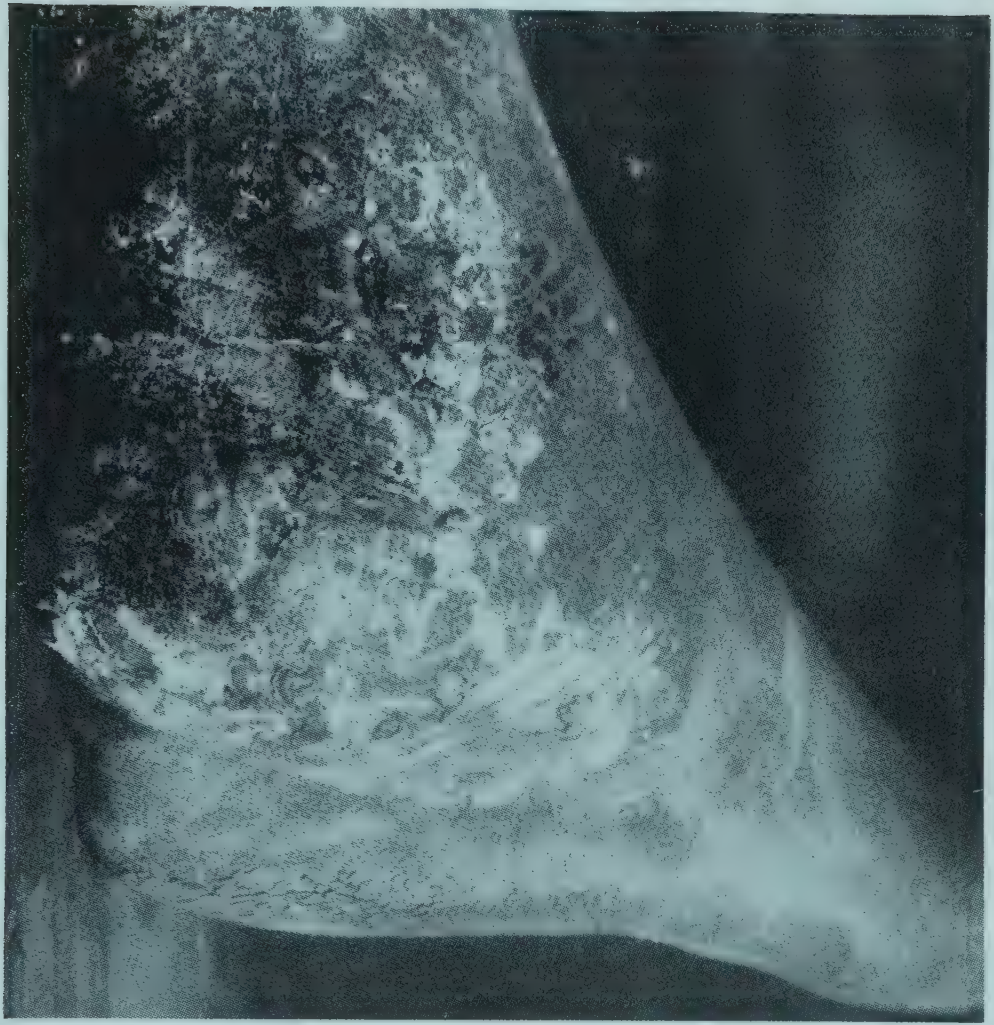


FIGURE 101: Mycetoma pedis (Reproduced from: Pijper, A. and Pullinger, B. D. J. Trop. Med. Hyg. p. 2, June 15, 1927).

Actinomycosis

An extensive literature has accumulated, since the early work of Bollinger and Harz, on the etiology of actinomycosis. Among the more recent investigations are the work of Chiarolanza (1910), Harbitz and Grondahl (1911), Klinger (1912, 1921), Galli-Vallerio (1912), Lignières and Spitz (1924), Magnusson (1928), Feit (1928), Naeslund (1929), Lord (1933), Grooten (1934), Lord and Trevatt (1936), Mohler and Shahan (1937), Lentze (1938, 1948), Emmons (1937), Cope (1938), Gins and Paasch (1940), Davis (1941), Slack (1942), Thompson (1950), Gonzalez-Ochoa and Sandoval (1955), and many others dealing especially with the occurrence of actinomycetes in connection with special infections.

Actinomycosis in animals was discussed by Lord (1910), Sforza (1940), and various others. The use of animals as diagnostic aids for the identification of *A. bovis* has been discussed by Meyer and Verges (1950).

In general, from a historical point of view, our concepts of the nature of the organisms that cause actinomycotic infections in men and in animals are closely related to the development of our concept of actinomycetes in general. The animal pathogen *Actinomyces bovis* has contributed the name "actinomycetes" to the whole group of these organisms, "Actinomycetales" to the taxonomic order, and "actinomycosis" to the major disease. A very extensive literature has accumulated on the pathogenic nature of actinomycosis; the identity of the specific agent has been the subject of much speculation.

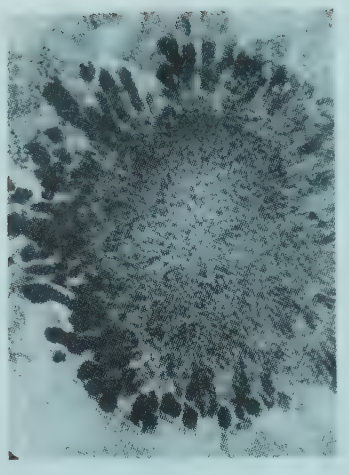


Figure 102. Granules and clubs of *N. pretoriana* (Reproduced from: Pijper, A. and Pullinger, B. C. J. Trop. Med. Hyg. p. 2, June 15, 1927).

The specific disease affects both man and cattle, usually involving the jaw. Thus, the expressions "lumpy jaw" and "pig jaw" are frequently used to designate this condition. The disease is not contagious, but once acquired, it is difficult to eradicate. It is characterized by a swollen jaw and a hard board-like induration, accompanied by destruction of the normal tissue and formation of granulation tissue. "Sulfur granules" are frequently present in the pus. They consist of cellular debris and of radially arranged hyphae; these terminate at the periphery in the form of "clubs," which are composed of eosinophilic material forming a sheath around the hyphal tip.

Emmons emphasized that while various actinomycotic infections give rise to clubs, certain forms of the disease caused by actinomycetes, notably by *N. asteroides,* do not form such clubs (see Gibson). Even *A. bovis* may fail to produce clubs in some tissues and under certain conditions. Emmons, therefore, defined actinomycosis as "an infection caused by invasion of the host by some species of *Actinomcyes.*" Several forms of the disease were recognized: actinomycosis of the skin, actinomycotic meningitis, lung infection, actinomycotic types of mycetomas (Kanthack), although some of these may be more properly classified with "nocardiosis." The presence of sulfur granules is frequently considered as a diagnostic symptom of actinomycosis. Granules may be produced, however, by various organisms, whereas some actinomycetes do not form any granules.

As pointed out previously, various attempts to isolate the causative agent yielded aerobic cultures that were found later to be air contaminants. Wolff and Israel are credited with being the first to isolate from maxillary actinomycosis in cattle a culture which they found to be a microaerophilic form. This culture was identical with *A. bovis.* Minute pinpoint and dewdrop-like colonies appeared on the surface of anaerobic agar slant cultures. The colonies gradually became larger and formed ball-like, irregularly rounded, elevated nodules; they did not become confluent and homogeneous. Some of the colonies presented a prominent center with a lobulated margin, appearing in the form of rosettes. In stab cultures, growth was more pronounced and was limited to the lower portion of the line of inoculation. In liquid broth, small white flakes appeared under aerobic conditions, some floating on the surface and some falling to the bottom of the tube. In general, anaerobic conditions were favorable to the growth of the organism.

Microscopic examination of the culture grown on agar showed long filaments forming a network. These were arranged more or less radially; they were straight, wavy, or spiral, and sometimes branched. Smear preparations gave short, homogeneous, usually straight, but also comma-like, rods of varied length and width; the ends of the rods often showed club-like swellings.

The tumor-like growths of infected animals were situated partly on the abdominal wall and partly on the intestines, in the liver, and in other tissues. Microscopic examination showed typical actinomyces colonies. The histological appearance of the tumors was similar to that of actinomycotic tissue.

In 1905, Wright made a detailed study of actinomycosis in man and in animals. He suggested that the word "actinomycosis" be restricted to a suppurative process combined with granulation tissue formation, the pus of which contains the characteristic granules. These are made up of dense aggregates of branched filamentous microorganisms and of their transformation or degeneration products; the latter include the characteristic club-shaped bodies radially disposed at the periphery of the granule. Cultures isolated from human and bovine cases were found to show insufficient difference to justify their classification as separate species. Wright further suggested that organisms different from *A. bovis* and which were associated with other forms of actinomycosis be grouped together under *Nocardia*, and that those cases of undoubted infection caused by them should be designated as "nocardiosis."

The presence of actinomycetes in sputum and in the contents of carious teeth was studied by Lord. Emmons also obtained organisms of the *A. bovis* type from the oral cavity. He isolated from tonsils two microaerophilic types of actinomyces: one, morphologically and physiologically similar to *A. bovis*; and another somewhat different morphologically, but also considered as a strain of *A. bovis*. Slack differentiated between the exogenous and endogenous types of infection in actinomycosis: in the first, awns of grass and grain frequently observed in actinomycotic lesions suggested their role in the infection; in the second, the anaerobic organisms isolated from normal mouth, from tonsils, from carious teeth, and from pyorrhea pus suggest their etiology. The oral cavity was looked upon as the source of infection, possibly accompanied by sensitization. Numerous investigators (Magnusson, Negroni and Bonfiglioli) reported considerable variation in the strains isolated from different forms of clinical actinomycosis.

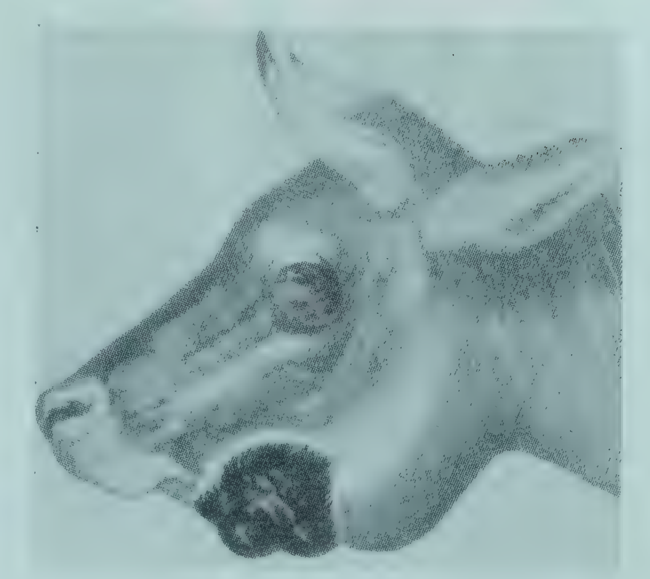


FIGURE 103. Lumpy jaw in a cow (Reproduced from: Mohler, J. R. and Shahan, M. S. U.S.D.A. Circ. No. 438, 1937, p. 4).

Maxillary actinomycosis is believed to be caused by organisms living in the mouth, since the contents of the mouth and tonsils were found capable of causing actinomycosis in experimental animals. It was suggested that *A. bovis* is often present in oral cavities, where it may exist as a saprophyte. Emmons also suggested the possibility that there are atypical strains found in certain lesions, in sputum, in carious teeth, and in tonsils. Human actinomycosis as influenced by mode and source of infection has been studied by Acland (1886), Shiota (1909), Mattson (1922), Shapiro (1931), Thompson (1950), and numerous others.

Actinomycotic endocarditis has been studied by Wedding (1947); actinomycosis of the eye by Herrenschwand (1927), of the face and neck by Lamb *et al.* (1947), by Glahn (1954), and by others. Skin actinomycosis has been studied by Namyslowski (1909, 1912) and Daines and Austin (1932); actinomycosis of the knee by Moore *et al.* and many others; liver abscess by Bloomfield and Bayne-Jones (1915); actinomycosis of the esophagus by Langer, of tonsils by Davis (1914), of the oral cavity by Naeslund (1925), and Sullivan and Goldsworthy (1940), cervicofacial by Glahn (1954), of the nervous system by Jacobson and Cloward, and of the heart by Cornell and Shookhoff (1944). Pulmonary actinomycosis has received much attention (Warthin and Olney, 1904; Sartory and Sartory, 1925; Penta, 1941; Lynch and Holt, 1945; Poppe, 1946; Vawter, 1946; Garrod, 1952). The earlier literature on various forms of actinomycosis is found in the work of Schlegel (1928).

Erikson suggested that the anaerobic organisms should be divided into the human and the bovine types. Lentze also concluded that actinomycosis in man and in animals represents two different types of anaerobic gram-positive organisms. Those involved are: (a) one (R-type) capable of growing on the surface of the medium, forming leathery irregular colonies and producing a sediment in liquid media; (b) another (S-type) producing smooth, easily broken colonies on solid media and causing turbidity in anaerobic liquid media. The first represents the classical type of Wolff-Israel and the other resembles corynebacteria. According to Wright, however, no significant differences exist between human and bovine strains.

Gradually, an extensive amount of literature has accumulated on the etiology of infections caused by actinomycetes. Naeslund grouped these infections under the anaerobic and aerobic types. The first, or A form, can be readily isolated from the mouth; it is a typical *A. bovis* and can bring about the true actinomycotic infection. The second, or B form, is a pathogenic aerobe, considered to be less important than the anaerobe; it is commonly found in nature, usually producing reddish or yellowish colonies, is acid-fast, and usually forms spores; it comprises the forms now included under *Nocardia*.

Rosebury isolated four strains of *A. bovis* from cervicofacial actinomycosis, and 11 from gingival scrapings taken under oral pathological conditions in the absence of actinomycosis. Optimum conditions for growth of these organisms were provided by anaerobiosis in the presence of 5 per cent carbon dioxide. Considerable variation was observed in oxygen tolerance among the different strains at different times. Pure cultures were maintained by cultivation under anaerobic conditions. Cope summarized the data of 1330 cases of actinomycosis: of these, 56.8 per cent affected the cervicofacial region, 22.3 the abdomen, 14.9 the thorax, and 5.9 per cent other sites.

The clinical features of actinomycosis were described by Conant and Rosebury as follows:

> "Actinomycosis is a subacute or chronic, generally progressive disease of man, cattle, swine, horses and other animals, characterized by the development of indurated granulating swellings chiefly in connective tissue, by suppuration usually of limited extent, and by the presence in the pus or lesions of *Actinomyces bovis*, demonstrable microscopically or culturally. In man the lesions are found chiefly in the cervicofacial connective tissues and in the thoracic or abdominal viscera, and develop over periods ranging from a few weeks to a year or more. The lesions spread widely by contiguity, sometimes pointing toward the skin and forming fistulae that tend to heal and reform elsewhere; rarely pointing toward mucous or serous membranes. The organism may be disseminated through the blood, or, in the lungs, through the bronchi. The lymphatic system is only rarely involved. Bone may be eroded in the path of the lesion, but is seldom affected interstitially except in the jaws."

Different forms of actinomycosis are further differentiated by Conant and Rosebury:

> "Cervicofacial actinomycosis accounts for more than half of all cases in man. It apparently originates from the mouth, but affects the soft tissues and skin of face and neck, and the tongue, and secondarily, the maxillary bones. The salivary glands, larynx, thyroid, and lacrymal glands, the orbit and even the brain may more rarely be involved. The commonest lesions appear on the

cheek or submaxillary skin, and are characterized by indurated or edematous swellings, bluish or reddish in color, with a tendency to form a series of irregular folds separated by furrows, the healing lesions forming scars as new lesions develop.

"Thoracic actinomycosis accounts for about fifteen per cent of human cases. It is found mainly in the lungs, with the formation of abscesses and cavities which are usually small. Extensive lesions may be found in the bronchi, and their rupture may lead to dissemination of the infection by way of the bronchial tree. Actinomycotic pleurisy and empyema have been observed, as has involvement of the heart and pericardium. Thoracic lesions may originate from the mouth or throat by aspiration, by extension from the abdomen, or by metastasis.

"Abdominal actinomycosis comprises about twenty per cent of human cases. The lesions may be found in any organ but are most common in the region of cecum and the appendix. From here they may extend with suppurating foci and the formation of fibrous adhesions to the abdominal wall, where skin lesions may appear similar to those of cervicofacial actinomycosis. Or the lesion may remain circumscribed, forming a fibromalike mass. The liver is commonly attacked, and lesions of the genital tract are relatively frequent. The stomach, small intestine and kidney are seldom affected. Infection is probably derived in most instances directly or indirectly from the intestinal or genital mucous membranes which, however, are not themselves involved. In the skin, actinomycosis, secondary to lesions of underlying tissues or organs, is relatively common, as has been noted; but it is doubtful whether true actinomycosis is ever primary in the skin."

Nocardiosis

Nocard was the first to describe, in 1888, a pathogenic actinomyces of the aerobic type. This organism was found to be the cause of "farcin du Boeuf," a disease of cattle in the Guadeloupe Islands. Trevisan named this organism *Nocardia*, in honor of its discoverer, the species being *N. farcinica*. Soon afterwards, Eppinger described a filamentous organism found in the pus of a cerebral abscess; he designated it as *Cladothrix asteroides*. This organism was transferred to the genus *Nocardia* by Blanchard and Nocard (1896). MacCallum reported in 1902 that *N. asteroides* produces a diffuse peritonitis in experimental animals. According to Benbow, Smith, and Grimson, about 90 per cent of all clinical cases of actinomycosis are caused by *A. bovis*; the remaining 10 per cent are caused by *N. asteroides*, most strains of which are partially acid-fast. Benbow *et al.* excluded from this classification the mycetomas which are caused by other species of *Nocardia*.

Following these early studies, much work was done on nocardiosis and the organisms involved. It is sufficient to mention that of Nakayama (1906), Evans (1918), Drake and Henrici (1943), Gonzalez-Ochoa (1945), Gonzalez-Ochoa and Hoyos, Binford and Lane (1945), and Kirby and McNaught (1946).

Pijper and Pullinger (1927) emphasized the affinity for iron among the *Nocardia* organisms.

Henrici differentiated between three well-defined types of infection caused by actinomycetes in man and in animals: 1. The lumpy jaw type, which is the most common infection and is produced by an organism belonging to *A. bovis* studied by Israel. 2. The madura foot type, caused by an aerobic form which is usually designated as *Nocardia madurae*, and more recently recognized as *Streptomyces madurae* (Mariat, 1958). 3. A rare type of infection caused either by *N. asteroides*, most frequently in man, or by *N. farcinica*, which occurs in cattle. Glover *et al.* (1948) suggested that the term "actinomycosis" be restricted to infection due to the microaerophilic *A. bovis* and that "nocardiosis" be used for infections caused by the aerobic *N. asteroides* and other species of *Nocardia*.

As many as 13 species of *Nocardia* isolated from white, yellow, or red granules found in the pus in cases of mycetoma have been described, but some of these names are now recognized as synonyms. Vincent cultivated the organism now known as *S.(N.) madurae.*

He considered it to be the most common causative agent of the disease. These aerobic organisms cause specific types of mycetomas. Infections of the lungs and of the skin are frequently produced. The organisms are cultivated much more readily than the anaerobic types and are pathogenic to laboratory animals. *N. farcinica*, isolated from cattle, forms a yellowish, wrinkled growth on solid media. *N. caprae*, isolated from the lung of a goat, gives a more whitish growth and greater fragmentation of the mycelium. *N. canis*, which produces infection in dogs, is similar to *N. caprae*.

Conant and Rosebury described the clinical features of nocardiosis as follows:

> "Nocardiosis is a chronic suppurative, purulogranulomatous disease of the subcutaneous tissues and bones (mycetoma) characterized by multiple tumefactions and draining sinuses from which granules (yellowish-white, red, or black) are expressed in the pus or found in the tissues; or, a pseudotuberculous infection (systemic) of the lungs and pleura with hematogenous spread throughout the body, especially to the brain and meninges, in which filamentous, bacillary or coccoid, acid-fast forms may be found in the sputum, spinal fluid, or pus from subcutaneous abscesses.
>
> "Mycetoma of the extremities results in the clinical picture of Madura foot, although other subcutaneous tissues of the body also may become infected. The characteristic lesion with pain, swelling, and sinus formation, and eventual clubbing and marked deformity of the infected member is developed only after months or years. Infection spreads by extension through adjacent tissues with bone destruction, multiple abscesses with rupture, and with no systemic reaction unless secondary bacterial invasion is established. Histologically, sections of the sinus and abscess walls may show only a chronic inflammatory reaction. Further development of the acute purulent abscess results in a surrounding layer of granulation tissue infiltrated with round cells and fat-laden macrophages enclosed by a fibrous capsule. Diagnosis, however, depends on the presence of granules, surrounded by polymorphonuclear neutrophils, centrally located in the abscesses.
>
> "Systemic nocardiosis is caused by *N. asteroides* and is chiefly pulmonary in origin. Of thirty-four cases, including two new cases, reviewed by Kirby and McNaught (1946) the lungs were infected in twenty-nine and, of these, eleven had metastases to the brain. Occasionally the presenting symptoms of headache, nausea and vomiting may indicate either brain tumor or brain abscess; or, the symptoms may be those of an infectious meningitis (tuberculous) with minimal or no findings in the lungs. Symptoms referable to a pulmonary infection include general malaise, fever, productive cough with sputum, night sweats, anorexia and loss of weight. Roentgenograms of the lungs usually show a progressive infiltrative process which may lead to multiple cavity formation. Hematogenous spread results in metastatic lesions throughout the body. Histologically, such lesions may be of a purulent nature, containing centers composed of polymorphonuclear neutrophils and a few mycelial fragments, or such areas may show a more advanced granulomatous reaction leading to granulation tissue, giant cells and scarring."

Granules are not formed by *N. asteroides*.

Mariat (1957) made a detailed study of the causative agents of chronic subcutaneous lesions, known as mycetomas. Only five species were recognized:

1. *Nocardia asteroides.* An infrequent causative agent of mycetoma. Semi acid-fast organism. Enzymatic reaction reduced or absent; pathogenic to experimental animals.
2. *Nocardia brasiliensis.* Prevalent in Central and South America. Semi acid-fast. High enzymatic potential. No agreement on experimental pathogenicity.
3. *Streptomyces madurae.* World-wide distribution. Causative agent of mycetoma. Forms large granules, white to reddish white; surrounded by long, club-shaped swellings. Hyphae not acid-fast. Nonpathogenic to animals.
4. *Streptomyces pelletieri.* Found largely in Africa. Granules small, numerous, and red, often fragmented. Hyphae not acid-fast. High enzymatic potential, but not pathogenic to animals.
5. *Streptomyces somaliensis.* Frequently found in Africa. Granules yellowish white. Hyphae not fragmented. High enzymatic potential. Not pathogenic to animals. The nu-

tritional properties of these organisms have been presented in Chapter 7.

Further analyses of nocardiosis, or the aerobic actinomycotic diseases due to aerobic actinomycetes, largely of the *Nocardia* type, are found in the numerous textbooks on bacteriology (Plehn).

Gruter made a comprehensive study of the occurrence of actinomycetes in eye infections, especially in tear glands and ducts. He came to the conclusion that a nocardia was involved. He designated his culture *A. discofoliatus.*

According to Gordon and Hagan, certain acid-fast actinomycetes isolated from soils and plant material are similar to those found in lesions of men and animals. The pigments produced by these organisms range from yellow through orange to coral. One of the soil forms, soon after isolation, was found to be pathogenic to rabbits but not to guinea pigs.

Allergic Reactions

Various attempts have been made to examine the immunological reactions of actinomycetes. Goyal compared 11 cultures obtained from collections and as fresh isolations. Most of them appeared to be members of the genus *Nocardia*. When inoculated into rabbits, they proved to be either entirely nonpathogenic or only slightly virulent, except for *N. eppingeri*. The cultures were grown in glycerol broth, at 38°C for 30 days; extracts, designated as "streptothricin," were prepared in a manner comparable to tuberculin (Helzer). Animals sensitized to the nocardia extracts ("nocardin") were also sensitive to tuberculin, and vice versa (Bletey). Serologic studies confirmed the conclusions reached on the basis of allergy tests; a common antigen was demonstrated for the tubercle bacillus, the diphtheria organism, and the nocardias. These results led to the conclusion that there is a definite antigenic relationship between the actinomycetes and the mycobacteria.

Further studies of the allergic reactions of actinomycetes have been made by Mathieson *et al.* (1935).

Therapy of Actinomycotic Diseases

The therapy of actinomycosis has been given rather limited consideration (Menning, 1933; Heuber, 1940).

According to Cope:

> "The prognosis of actinomycosis varies greatly according to the part of the body affected. It is most favourable with those cases which affect the head and neck, less favourable with abdominal cases, and most unpromising with thoracic disease
>
> "The great majority—probably 97 per cent—of those cases in which the cheek, jaw, tongue, and neck are involved come to a satisfactory issue. The disease may last for several years but nearly always ends satisfactorily. The 3 per cent of cases in which death results are those in which the pathological process either extends deeply towards the base of the skull and causes cerebral complications, or extends downwards to the superior and posterior mediastinum, or more rarely, becomes generalized."

Treatment of actinomycosis consisted first of radiation therapy and use of vaccines. More recently, with the advent of the sulfa drugs (given internally or applied locally) and especially the antibiotics, chemotherapy of actinomycotic infections took a new turn. Previously use was made of specific vaccines (Scott), and iodine was considered to be by far the most important drug which had a definite effect in the treatment of actinomycosis (Cope). But today the sulfa drugs and the antibiotics have taken the place of iodine in chemotherapy, of both actinomycosis and nocardiosis. This was demonstrated by Cutting and Gebhardt (1940), Dobson *et al.* (1941), Hollenbeck and Turnoff (1943), Lyons *et al.* (1943), Hendrickson and Lehman (1945), Farris and Douglas (1947), Kay (1947), Holm (1948), Benbow *et al.* (1949), Boand and Novack (1949), Strauss *et al.*

(1951), Fischer and Harvey (1956), Bianco *et al.* (1957), and numerous others.

Among the antibiotics used were penicillin (Dobson and Cutting, 1945; Drake, 1946; Holm, 1948; Nakhimovskaia *et al.*, 1957), streptomycin (Pemberton and Hunter, 1949; Torrens and Wood, 1949), chloramphenicol (Littman *et al.*, 1952), the tetracyclines (Martin *et al.*, 1956; Lane and Kutsaber, 1956), and various others (Banerjee *et al.*, 1954; Hanf, 1956).

In a comparative study of the inhibitory effect of antibiotics upon the growth of the anaerobic *A. israeli*, Garrod found penicillin to be active in a concentration of 0.1 unit and streptomycin in 23.7 units, with the tetracyclines and chloramphenicol falling between (2.2 to 4.2 units). This bears out the sensitivity of anaerobes to penicillin and their relative resistance to streptomycin. Frequently an antibiotic is active only *in vitro* or *in vivo*. The most effective antibiotics against *N. asteroides*, for example, were found to be, when tested *in vitro*, erythromycin and novobiocin; however, the only therapeutic action in experimental animals was exerted by cycloserine.

Mackinnon *et al.* made a detailed study of the effect of various chemotherapeutic agents upon mycetoma and nocardiosis. They found that strains belonging to the same species show sufficiently similar susceptibility as to prove species sensitivity. Three species of *Streptomyces* (*S. somaliensis*, *S. pelletieri*, and *S. madurae*) were susceptible to at least two of the following antibiotics: penicillin, streptomycin, chlortetracycline, and chloramphenicol. These species could be distinguished by their relative sensitiveness to these antibiotics. Diaminophenylsulphone is active upon *N. brasiliensis*, *N. asteroides*, and *S. somaliensis*, but less so upon *S. pelletieri*. The pathogenic actinomycetes were found to show development of resistance to streptomycin and to aromatic diamidines.

CHAPTER 18

Causation of Plant Diseases

Very few forms among the actinomycetes are capable of causing plant diseases. In spite of the great abundance of actinomycetes in nature, especially in the soil, the number of plants attacked by them, as compared to the number of plants attacked by bacteria, fungi, and viruses, is rather limited. Except for two species—the Irish potato and the sugar beet—plants subject to infection by actinomycetes do not occupy a very prominent place in human economy.

Potato Scab

An extensive amount of literature has accumulated dealing with the causation of potato scab, the development of the disease, the organisms involved, the effect of environment, and methods of control. Our main concern here is the causative agents of the disease.

Organisms

The organisms capable of causing common scab of potatoes were at first believed to comprise only a single actinomycete. Gradually it came to be recognized that a number of species, or at least a number of races or strains, all belonging to the genus *Streptomyces*, are capable of causing the infection.

In 1890, Thaxter first described the organism causing potato scab under the name *Oospora scabies*. This was later changed to *Actinomyces chromogenus*, then to *Actinomyces scabies* (Güssow), and finally to *Streptomyces scabies* (Waksman and Henrici). Because of its historical significance, the description given by Thaxter is quoted here:

> "Vegetative hyphae brownish, .06 [0.6?] to 1.0 μ in diameter, curving irregularly, septate or pseudoseptate, branching. Aerial hyphae at first white, then gray, evanescent, breaking up into bacteria-like segments, after having produced single terminal spiral spores by the coiling of their free extremities. Forming a firm lichenoid pellicle on nutrient jelly and usually producing a blackish-brown discoloration of the substratum on which it grows, causing the disease known as scab on potato tubers, and a similar disease of beet roots."

The above description fits in perfectly well with that of a typical species of *Streptomyces*.

Thaxter's belief that a single organism is concerned with potato scab, as well as with sugar beet scab, prevailed for many years. More recent studies of cultures of actinomycetes isolated from various types of scab suggested the probability that several species are involved in the formation of scab. Most investigators now tend to believe in the multiple origin of this disease. It has even been suggested that many actinomycetes found in the soil have pathogenic tendencies which they may lose, if the host is not present. If a suitable host is provided, the pathogenic habits are reacquired. On the other hand, some investigators still believe that not only is the organism causing potato scab a single species, but that *S. scabies* can cause root necrosis in seedlings of wheat, pea, soybean, radish, and a variety of other plants of many families.

Wollenweber made a comprehensive study of the organisms concerned in the causation of potato scab. He was the first to suggest that more than one actinomycete has the capacity to cause scab. He also emphasized that different types of scab are caused by different organisms. No infectivity tests on potatoes were made, however, with the isolated cultures. Further, Wollenweber's descriptions were incomplete and the experimental data presented in support of his assumptions were limited. Since most of the cultures isolated from scabby potatoes thus described are similar to the large numbers of saprophytic organisms commonly found in the soil and on surfaces of potatoes, the pathogenicity of the species believed to cause scab is open to question.

Millard and Burr isolated from potato scab a large number of cultures of actinomycetes believed to be responsible for the causation of the disease. Only three of these cultures were obtained in duplicate, and only one was found to be identical with Thaxter's original isolate. The latter produced the deep form of scab and was capable of attacking the roots and stolons of the potato plant. Millard and Burr believed that different types of scab were caused by different organisms. They used glycerol synthetic solution as the basis for growth of their cultures.

The mere isolation of an organism from a scabby potato is no proof, however, that the organism is capable of causing the disease. This has been emphasized by Decker and numerous others.

DeBruyn studied in detail the specific nature of scab-producing actinomycetes. He employed the method described by Kiessling. Sap of different varieties of potatoes is added to synthetic media and inoculated with actinomycetes. Since the pH of the sap changes with the ripeness of the tuber, the growth of the organisms in such media is parallel to the pH change. The cultures made their best growth in the presence of sap of a susceptible variety; no growth occurred in a medium to which the sap of young tubers of a resistant variety had been added. DeBruyn was able to confirm the ideas of Wingerberg and Kieszling concerning the existence of physiological scab resistance. Four types of scab were recognized: (a) deep, (b) tumulus, (c) common, and (d) superficial or russet scab; each type was found to be caused by a different species. All of them are now recognized as belonging to the genus *Streptomyces.*

Leach *et al.*, however, could not confirm the reports of the multiplicity of scab-producing actinomycetes. On the basis of their studies, they emphasized that scab lesions are a result of a reaction between the pathogen and the host tissue; they may be influenced as much by the host as by the pathogen. They recognized, however, the existence of pathogenic races of the organism. Cocchi's results, on the other hand, tended to support, at least partly, Millard and Burr's conclusions; two of the three strains he isolated were identified as *S. flavus* and *S. clavifer.*

Taylor and Decker isolated 143 cultures of actinomycetes of which 128 were nonacid-fast and 15 partly acid-fast. Starch was hydrolyzed by all of the nonacid-fast isolates and by none of the partly acid-fast isolates. The ability of the cultures to produce a dark brown surface ring of growth on milk was characteristic of 66 isolates, all nonacid-fast. Of the remaining 77 cultures, 71 produced on alkaline reaction; four formed a hard, acid curd; one showed no visible change in reaction in the presence of abundant growth; one did not grow at all on milk.

Thirteen of the acid-fast isolates gave microaerophilic growth on agar-shake tubes. A dense ring of colonies developed beneath the surface of the agar, at depths of 5 to 10 mm. As the cultures grew, additional rings developed beneath the original one. With the nonmicroaerophilic isolates, no growth was produced below a depth of 5 mm; any subsurface development was continuous with the surface growth. Gelatin was liquefied by all of the nonacid-fast cultures and by the two nonmicroaerophilic acid-fast forms. The 13 microaerophilic acid-fast isolates did not liquefy gelatin.

Typical potato scab, as distinguished from superficial russetting of the surface, was produced by all of the "dark brown ring" isolates; none of the other isolates that were tested caused typical scab on potato tubers. Within the first group, variations occurred in the amount of scab formed on the tubers, though all produced some scab infection. The particular cultures did not differ consistently in type of scab lesion produced. In many cases scab lesions varying from shallow depressions to deep pits were found among the replicated pots of one isolate, and frequently within the same pot or on the same tuber.

Superficial russetting was not considered as typical of potato scab. It was more abundant with certain isolates in the nonacid-fast group. This russeting was not observed in the tests of the "dark brown ring" group. The correlations observed between pathogenicity and the behavior on milk held true for a large number of actinomycetes. The conclusion was reached that the ability to produce typical lesions of potato scab was correlated perfectly with the production of a dark brown ring of surface growth on separated milk.

Afanasiev isolated seven cultures of the scab-producing organism from three different types of scab: common, deep, and russet. Individual potatoes were found to show two or even all three forms of scab. The difference in the kind of scab caused by cultures of actinomycetes was believed to be one of degree of pathogenicity rather than of type of organism involved. Certain marked physiological differences were found between the parasitic and the saprophytic cultures. The parasites were able to utilize sucrose and raffinose; most of the saprophytes were unable to use these two sugars. The growth of both types of cultures was similar on all media containing different nitrogenous compounds, with the exception of urea; the parasitic and some of the saprophytic cultures failed to grow on a medium to which 0.5 per cent of urea was added. This was found to be due to the toxicity of the ammonia produced as a result of the decomposition of the urea. On the basis of their physiological and biochemical properties, namely, ability to utilize sucrose and raffinose, inhibition of growth by ammonia, and ability to produce a melanin pigment in a tyrosine medium, parasitic cultures could be differentiated from ordinary saprophytic actinomycetes.

Finally, it may be of interest to report here the recent work of Hoffmann (1958). Numerous infection experiments were carried out with twenty *Streptomyces* species, using a number of scab-susceptible potato varieties in the greenhouse and under field conditions. He found that *S. scabies* was the sole pathogen of potato scab; the same was true of beet scab. He regarded the "parasitic" forms of scab reported by other investigators as saprophytic organisms accompanying the actual pathogen.

Environment

The nature of the soil in which the potatoes are grown and the environment play the greatest role in the production of scab. The organic matter content of the soil, and its degree of decomposition, the inorganic structure of the soil, and the soil reaction (Gillespie) are among the most important factors in this connection. The environmental conditions comprise moisture, temperature (Jones *et al.*), and aeration.

According to Dippenaar, the optimum temperature for scab infection, as determined by the average number of lesions per tuber, was found to be between 17 and 21°C. The optimum temperature for the development of the disease was about the same as the one best suited for the host, and did not

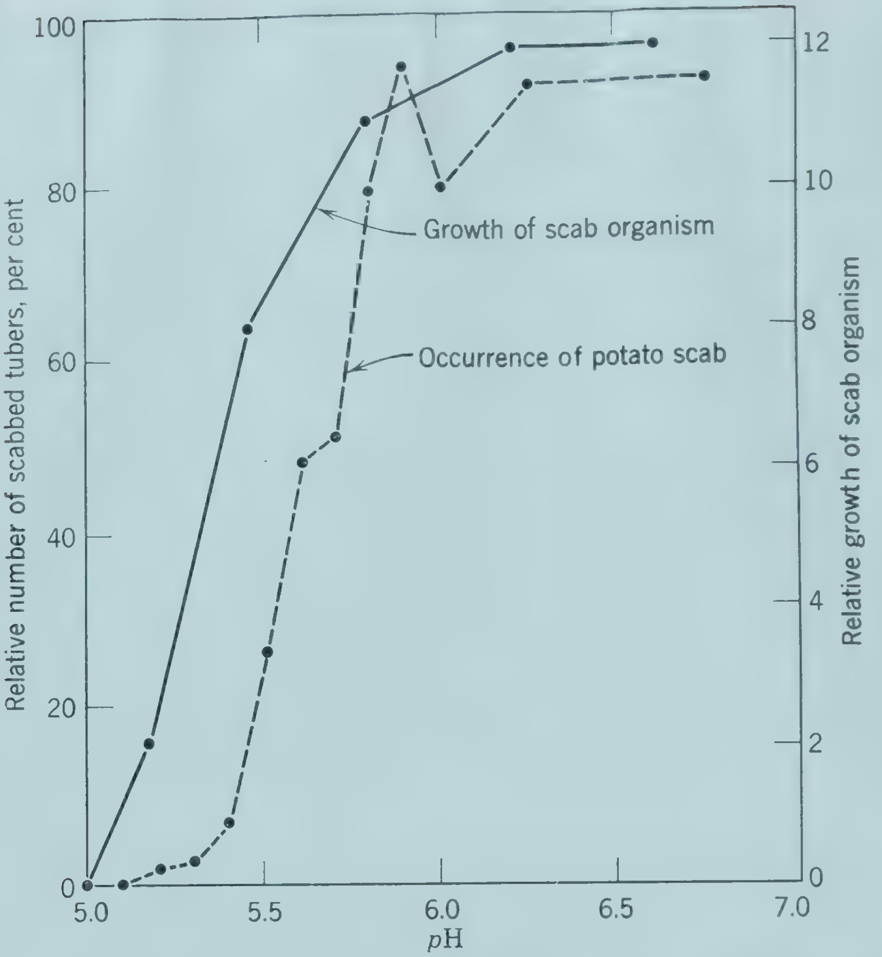


FIGURE 104. Influence of pH on the growth of the potato scab organism (Reproduced from: Dippenaar, B. J. Union of South Africa Dept. Agr. Sci. Bull. 136: 68, 1933).

vary with the soil moisture or the soil reaction. Increasing soil moisture decreased the amount of scab at all soil temperatures and in all types of soil used, and increased the yield of potatoes. Lower moisture was required to control scab at 13 and at 25°C than at either 17 or 21°C. The disease on actively growing tubers was controlled by increasing the soil moisture after the tubers had developed scab. Relatively scab-free tubers growing in a scab-infested soil with a high soil moisture content will become scabby if the soils are allowed to become dry.

In greenhouse experiments, Dippenaar found that a reaction of pH 5.0 and lower either controlled or reduced the disease in severely scab-infested soils but did not eliminate scab entirely. A reaction of pH 4.8 and lower, however, had an adverse effect on the potato plant.

Goss (1937) made a detailed study of the environmental factors influencing the causation of potato scab by *S. scabies*. The soil was found to be the major source of infection. Although crop rotation reduced considerably the incidence of the disease, the fact that tubers in soil never before planted to potatoes could still show severe scab suggested that various other factors were involved which influenced the severity of the disease.

Among these factors, high temperature, low moisture, alkalinity, abundant aeration, and the addition of barnyard manure are generally accepted as favoring the incidence of the disease. On reading the extensive literature, one would assume that it should be possible to correlate the occurrence of the disease in the field with one or more of these factors. Unfortunately, it has often been

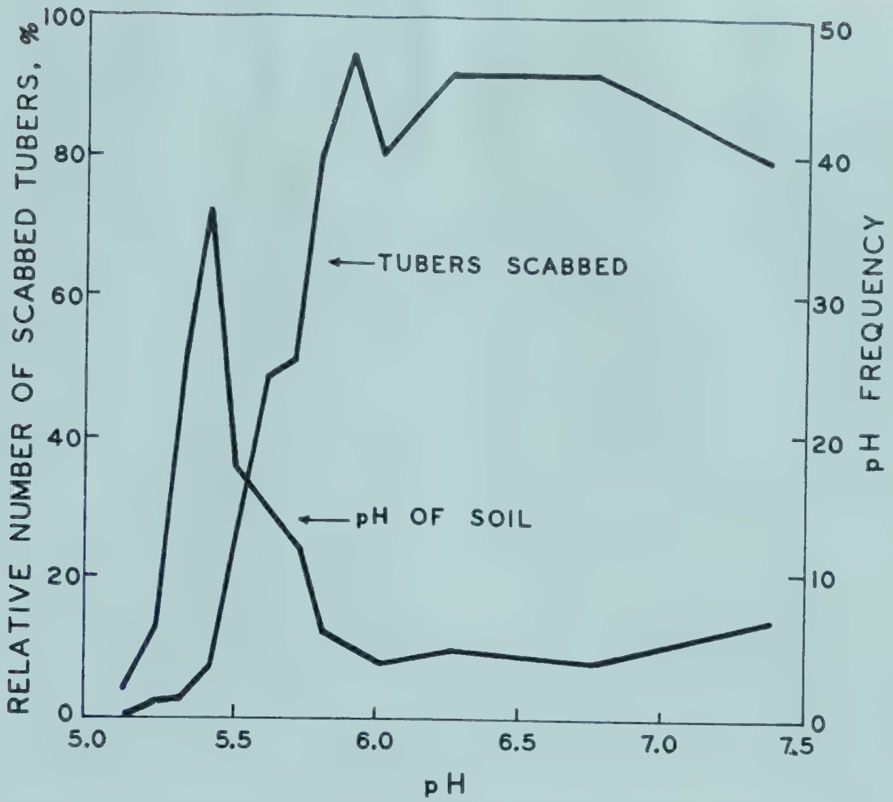


FIGURE 105. Relation of soil reaction to the occurrence of potato scab (Reproduced from: Dippenaar B. J. Union of South Africa Dept. Agr. Sci. Bull. 136: 52, 1933).

found difficult if not impossible to do this. It has been observed in western Nebraska that potato scab is always severe following heavy, packing rains when the field cannot be cultivated subsequently. Scab was also commonly found in flooded areas, at the lower ends of irrigated fields, and in poorly drained portions of dry-land fields. This is the reverse of what one might expect from the work of Sanford and Dippenaar on soil moisture and of Sanford on the necessity of abundant aeration for the best development of the scab organism.

A study was made to determine whether the effect of soil sterilization on the occurrence of scab was due to chemical or physical changes in the soil or to lack of competing organisms. Sterilized soil was treated with an extract of unsterilized soil. The effect of the time of inoculation was determined by adding the inoculum either at the time of planting or at the beginning of tuber formation. In sterilized soil there was a greater amount of scab. This was due to a lack of competing soil microorganisms that would antagonize the scab-producing forms. The numbers of actinomycetes in the soil, as determined by the plate method, checked well with the incidence of scab in the various tests. The largest number of actinomycetes, 82 days after sterilization, occurred in the soil which had been inoculated for the longest time and in which the inoculum had apparently become well established before the

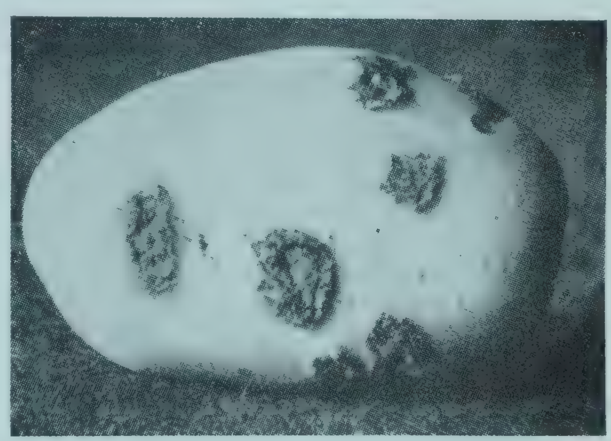


FIGURE 106. Corky scab of potatoes (Reproduced from: Millard, W. A. *Common scab of potatoes*. Univ. of Leeds Pam. No. 118, 1921, p. 2).

soil became infested with competing organisms.

Further tests of the effect of additions of sterilized and nonsterilized organic matter and of the filtrates of both to sterilized inoculated soil confirmed the above observations. Three types of organisms were isolated from the unsterilized soil, cultivated, and added to the soil in amounts approximately equal to the *S. scabies* content of the soil. This was for the purpose of determining whether any one of these particular groups was an effective competitor of the scab organisms in the soil. These tests were made during different years in the greenhouse, but the results were of the same general nature (Table 76).

TABLE 76

The effect of the competition of soil microorganisms upon the occurrence of scab in inoculated soils (Goss)

Treatment of soil*	Number of plants	Number of tubers	Tubers in various classes, determined by percentage of scabby surface: 0	0–2	2–25	25–100
Sterilized soil, inoculated with S. scabies						
1. No treatment	58	249	2	6	34	58
2. Filtrate of unsterilized soil	34	176	8	17	27	48
3. Filtrate of sterilized manure	40	205	7	8	36	49
4. Sterilized manure	20	102	12	13	49	27
5. Filtrate of unsterilized manure	39	215	20	22	34	24
6. Unsterilized manure	20	78	54	17	23	6
7. *Penicillium* sp.	30	160	1	4	35	60
8. Bacteria	30	160	9	9	38	44
9. *Streptomyces* sp. (saprophytic)	20	103	7	7	48	39
10. Mixture of treatments of 7, 8, 9	19	94	2	2	52	43
Sterilized soil, not inoculated						
11. No treatment	20	85	100	0	0	0
Unsterilized soil, inoculated with S. scabies						
12. No treatment	50	211	17	25	39	19

* All treatments were made at the time of inoculation. All inoculations were made at the rate of 200 ml of inoculum per pot, equal to 1⅓ Petri dish cultures. Saprophytic organisms were added at the same rate. The filtrates were obtained by soaking 4 parts of soil or manure in 6 parts of water overnight, filtering through cheesecloth, and adding 200 cc per pot. The manure in Sets 4 and 6 was added at the rate of 200 gm per pot.

Goss stated that in emphasizing the numbers of actinomycetes and their relation to soil moisture and other factors, one must consider the fact that these organisms must be growing vegetatively if they are to cause infection. High plate counts often indicate sporulation or fragmentation of the mycelium, whereas low counts may indicate vegetative development, and this in turn may be correlated with high infection. Dippenaar suggested that germination, growth, and sporulation may all occur at their maximum under similar conditions. Conditions favorable for sporulation would tend to increase the scab-producing power of the soil. If this were followed by conditions favorable for vegetative development, more infection might result. The effect of moisture and sterilization upon scab development is brought out further in Table 77.

Numerous other investigations have been carried out throughout the world on the effect of various environmental factors on growth of the potato scab organisms and on the causation of scab (Shapovalov, Janchen, Cocchi, Blodgett and Howe). The effect of the variety of potato on scab development also has received considerable attention (Umbreit, 1938).

Relationship of Host to Parasite

Schaal and his collaborators made an extensive study of the effect of phenolic substances upon the degree of infection of potatoes (pustule type). The formation of chlorogenic acid in the cells of the lenticel area was the causative mechanism (Johnson and Schaal, 1952; Schaal and Johnson, 1955; Schaal *et al.*, 1953). They found that, in cells adjacent to the periderm, chlorogenic acid was present in larger quantities in potato tubers of scab-resistant varieties than in those of susceptible varieties. A test that would not require destruction of the whole tuber was devised so the tested tuber could be used for seed purposes. The test was based on the fact that chlorogenic acid is localized in the cells directly under the epidermis and corky covering and in cells around the lenticels of the highly resistant varieties. Scab resistance was found to be associated with the amounts of chlorogenic acid present.

The test consisted in placing several drops of 2 per cent aqueous ferric chloride solution on the surface of a tuber and macerating with a stainless steel knife the tissue covered by the test solution. By pricking this area under a drop of ferric chloride solution, the presence of chlorogenic acid in or near the lenticels could be determined. The distribution of chlorogenic acid in the tuber was measured by spreading the ferric chloride solution over the freshly cut surface of a half of a tuber. In resistant varieties a green color reaction was found near the surface. In some highly resistant varieties the chlorogenic acid was present throughout the tuber, its greatest concentration being in the cells directly under the corky covering. The color reaction was found to be greater in the immature than in the mature tubers. Different varieties showed differences in color reaction.

The effect of six phenolic compounds on the growth of *S. scabies* was tested at pH 6.0, 7.5, and 8.5. Four orthodihydricphenols (chlorogenic acid, caffeic acid, catechol, and tetrahydroxybenzoin) were effective upon autoxidation in inhibiting the growth of *S. scabies* in culture medium. The inhibition increased with increases in the pH. These results were said to support the theory that the mechanism of scab resistance in potato tubers involves enzymatic oxidation of chlorogenic acid, which produces a quinone toxic to the scab organism.

The mechanism was believed to depend on the amount of chlorogenic acid in the periderm and on the presence of tyrosinase in the same tissue. The localization of this acid around the lenticels that serve as the normal entrance points for the scab organism was important. A tendency for the acid to accumulate in cells adjacent to injured areas was observed. Tyrosinase was found in high concentration in the tissue containing chlorogenic acid and was found to become oxidized when the tissue was injured. The oxidation products formed, such as quinones, were believed to be toxic to the invading organism.

Johnson and Schaal (1957) brought out further that the concentrations of chlorogenic acid and of total *o*-dihydricphenols were much higher in the periderm of tubers of certain scab-resistant varieties than in tubers of scab-susceptible varieties. The greatest difference between the varieties was found at the stage where the tubers were growing most rapidly. The tubers were analyzed at three stages of growth and after 5 months of storage at 35 to 38°F. The results indicated that the ferric chloride spot test for scab resistance could be made on immature or on freshly harvested mature tubers.

Effect of Treatment

Addition of stable manure to soil has usually been found to increase the incidence of scab, because of the resulting alkaline reaction and the accumulation of humus. Similar results were also reported from excessive use of potassium. Application of certain fresh organic materials, such as green manures, results, however, in the reduction of scab. Millard and Taylor saw in this effect a kind

TABLE 77

Effect of soil sterilization and moisture content on scab following inoculation with S. scabies (Goss)

Set No.	Soil treatment	No. of tubers	Weight of tubers, gm	Tubers in various classes determined by percentage of scabby surface*				
				0	0–2	2–25	25–75	75–100
1	Sterilized-high moisture	33	1460	1	4	35	34	26
2	Sterilized-low moisture	41	900	0	0	3	0	97
3	Not sterilized-high moisture	36	1795	3	12	84	1	0
4	Not sterilized-low moisture	38	1210	0	0	21	15	64

* Data based upon tuber weights.

of competition between the soil saprophytes and parasites. Further studies indicated that several factors may be involved. These comprise an increase in soil acidity, an increase in the buffering and moisture-holding capacities of the soil, and a possible stimulatory effect upon those soil microbes which exert an inhibitory effect upon the scab organisms.

Soil sterilization and subsequent inoculation lead to increased infection. This is apparently due, according to Goss, to a resultant lack of competition of soil microorganisms rather than to changes in the physical or chemical structure of the soil. Addition to such soils of a filtrate from unsterilized soil tends to counteract this effect; the effect of sterilization is greatly reduced if the inoculum is not added until after saprophytic organisms have become established in the soil.

Various other factors affect the pathogenicity of the scab organism, such as passage through the digestive tract of animals (Morse), spread by potato residues (Lutman), the influence of the potato variety (Longree), and the presence of antagonistic organisms (Daines).

According to Fellows, if the scab disease is to occur, the potato tubers must be increasing in size. Stomata or young unsuberized lenticels must be present through which the infection can take place. There must also be dividing cells or cells which can easily be incited to division by the products of the organism, thus permitting the production of the typical corky scab lesions.

According to Goss and Werner, seed treatments are effective in controlling seed-borne scab; however, even when healthy or treated seed potatoes are used, the disease may be very severe because of infection from the soil. Crop rotation reduces the incidence of the disease, but the fact that potato scab may cause serious loss in soils never before planted to potatoes suggests that other factors than the time interval between potato crops affect the occurrence of the disease.

Biological Control of Potato Scab

No attempt will be made here to review in detail the practical methods of control of potato scab (Berkner and Schröder, Noll). Suffice it to say that crop rotation (Werner *et al.*) and acidification of soil (Martin, Waksman, 1922, Duff and Welch, Cook and Nugent, Blodgett and Cowan) were found to be most effective. A complete review of the literature, especially of the environmental factors bearing on scab formation, has been made by Hollrung.

One of the most interesting aspects in the production and control of scab on potatoes is the possible effect of other soil microorganisms. Reference has already been made to the work of Millard. Sanford (1926) has shown that green rye plants, plowed into the ground at the rate of 50 tons per acre, showed no effect upon the reduction of scab in a well-infested soil at pH 5.0 to 5.4. There was no noticeable increase in soil acidity during a 58-day period. In artificial media, however the growth of certain bacteria made conditions unfavorable for the growth of *S. scabies.* The conclusion was reached that when scab is controlled in some soils by green manure crops, this may be due to the "antibiotic" qualities of certain predominant soil microorganisms.

KenKnight attempted, unsuccessfully, to control scab through the antagonistic activities of other microorganisms. Addition of organic matter to the soil tended to aggravate the development of scab except in extreme cases of scab infection. The findings of Millard and Taylor that green manure was effective in controlling scab in the presence of *S. praecox* were not confirmed. When this and other species of actinomycetes were introduced into scab-infested soil in green manure and in various other media no control was obtained. KenKnight thus agreed with Goss, who also failed to obtain control of scab with *S. praecox*.

McCormick observed that *S. praecox* was antagonistic on solid media to *S. viridis* and *S. intermedius*, but not to certain other parasitic actinomycetes. This observation suggested specific differences in the action of the antagonist. No control of scab was obtained with *B. megaterium*, an organism antagonistic to certain actinomycetes. *Pseudomonas fluorescens*, which was antagonistic to certain actinomycetes and to *Trichoderma lignorum*, reported to be antagonistic to many fungi, exerted no effect on the scab organism.

Daines reported (1937) that *Trichoderma lignorum* produces a diffusible substance which is toxic to *S. scabies* in an artificial liquid medium. Because of the rapid destruction of this toxic principle by aeration at the pH of potato soils and because of removal of the toxic material from solution by charcoal and by the soil itself, the efficacy of this fungus in combating potato scab was considered as doubtful. Although *Trichoderma* might be of some assistance in this capacity in poorly aerated soils which possess low adsorptive capacities, its function in many soils was questioned.

The physical as well as the biological environment in many cultivated soils was considered a strong barrier against the establishment of the *Trichoderma.* When introduced into a 5-day-old *S. scabies* culture, *Trichoderma* was strongly inhibited by the scab. A soil-inhabiting bacterium was also found to produce a material that was toxic to *Trichoderma* and to the actinomycete alike. Daines argued that "in such complex physical, chemical and biological environments, as are afforded by soils, these antagonistic relationships may be modified or even entirely destroyed."

KenKnight argued that the absence of proper biological controls in the above experiments may have been due to failure to establish the introduced organisms in the soil, or to the failure of these organisms to show antagonism toward any or all of the parasitic actinomycetes under soil conditions. He found an analogy between the introduction of organisms not well suited to the soil conditions and efforts to obtain a stand of wheat by planting the seed in unbroken prairie sod. He believed that Kiessling's mixed cultures of bacteria that had the capacity to control scab, except for one type of scab caused by a species other than *S. scabies*, suggested that if practical control of potato scab is ever obtained by biological means in soil infested with several parasitic actinomycetes, it will be with mixed cultures, because it appears unlikely that a single organism will be found that is antagonistic to all strains of the scab organism.

The relation of soil fungi to the development of potato scab has been studied further by Pratt (1918) and by others.

Detailed studies have been made of the relation of the soil population to the development of the scab organism. The question was raised whether actinomycetes diminish in numbers and whether the parasitic potato-scab-producing strains tend to die in soil in which no potatoes have been grown for a long time. The actinomycetes were found to remain fairly constant in their numbers and in their percentage relation to the total number of organisms. The pathogenic types, however, were gradually reduced in numbers. Tests were made by planting a susceptible potato variety in the soil; if the scab-producing strains were still present, scabbing would result. The formation of russetted tubers suggested the possibility that the scab organism, after a long period of deprivation of its host, was weakened in pathogenicity and produced the true, deep scab only in rare instances. *S. scabies* is also capable of causing necrosis of subterranean stems of potatoes. The stems may become girdled and rotted at the base with vascular discoloration extending up the stem six to eight internodes (Hooker and Kent).

Sugar Beet and Mangel Scab

Certain actinomycetes are also capable of causing scab on various root crops, notably sugar beets and mangels. Krüger was the first to establish, in 1904, that the production of scab on sugar beets is due to actinomycetes. Under the influence of the generic designation of the actinomycetes used by Thaxter, he described several actinomycetes as species of *Oospora*, namely, *O. cretacea*, *O. rosella*, *O. intermedia*, *O. tenax*, *O. nigrificans*, and *O. violacea*. All of these were, however, typical actinomycetes, belonging to the genus *Streptomyces*. Krüger studied particularly the type of scab known as "girdle" scab of sugar beets. The strains of the organisms he isolated were believed not to be identical with the potato scab form of Thaxter.

Various other isolations of actinomycete cultures were made from beet scab. Some of these cultures were found to be parasitic but varying in the degree of virulence. Millard and Beeley recognized two distinct types of mangel scab, the raised and the pitted forms. The raised scab was subdivided into the mound and knob types, which were found to develop particularly on yellow-skinned varieties of mangels. The pitted scab was similar to the common scab of potatoes, whereas the raised scab was not formed from the cambium of the vascular rings, but resulted from the proliferation of the pericycle. A culture was isolated from mound scab which reproduced the same type of scab in artificial inoculation experiments; it was described as *A. tumuli*. From pitted scab a culture was isolated which also reproduced

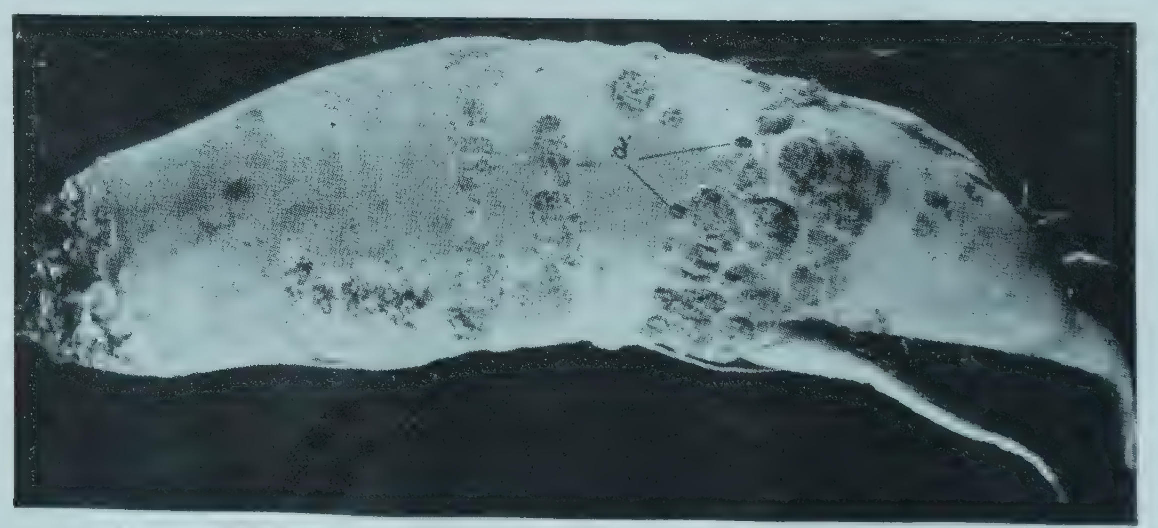


FIGURE 107. Mangel scab (Reproduced from: Millard, W. A. and Beeley, F. Ann. Appl. Biol. 14: 311, 1927).

its own type in inoculation experiments. This culture also attacked the roots and rootlets of the inoculated mangel plants, on which it produced numerous characteristic dark brown, nodular outgrowths. It was identical with *S. scabies.*

Sweet Potato Pox or Soft Rot

An actinomycete, designated by Taubenhaus as *A. poolensis,* was found to be a contributing factor to the causation of soft rot of sweet potatoes. It was considered as a superficial wound parasite, usually following the pox spots produced by a fungus.

Other cultures of actinomycetes were later isolated from sweet potatoes. One of them, designated as *A. pox,* was believed to be the cause of the pox disease (Adams).

Still another organism causing sweet potato rot was described as *S. ipomoea.* It produced aerial mycelium and was thus a member of the genus *Streptomyces.* Sweet potato rot does not develop in soils of pH below 5.2; above that reaction, the disease develops readily. According to Person and Martin, sweet potato rot is more serious in dry soils and in wet seasons than under normal moisture conditions. The disease has been produced in the greenhouse and in field inoculation experiments with pure cultures of *S. ipomoea.* The optimum reaction for growth is pH 5.6 or above, and the optimum temperature 32°C.

Other Plant Diseases Caused by Actinomycetes

A number of other plant diseases have been reported to be caused by actinomycetes. Banga described a strawberry disease. Godfrey listed a form causing citrus gummosis.

Hooker reported that seedling plants representing eight families developed root necrosis when grown in soil-extract agar artificially infested with pure cultures of *S. scabies.* Ten cultures of actinomycetes were tested on seedlings of wheat, garden pea, soybean, corn, radish, and cucumber, and on potato sprouts. Six of these cultures caused neither appreciable necrosis of potato stems nor injury to seedling roots. Four cultures caused severe necrosis of roots as well as a reduction in root weight of wheat, pea, soybean, and radish; necrosis was most pronounced on root tips, and the development of secondary roots was almost inhibited. Corn roots were only slightly necrotic, but their weight was markedly reduced. Three of the four cultures also caused scab of potato tubers and necrosis of potato stems.

Palm described the occurrence in South Sweden of actinomycotic infections of *Beta vulgaris, Brassica* sp., *Raphanus sativus,* and *Daucus carota.* He concluded that the infection of these plants may be caused by the same pathogen. The disease appears as sunken spots, delimited by the more prominent veins of the bulb scales, and are of a greenish mother-of-pearl-like color. The pathogen lives intracellularly, filling the cells completely with its very thin (1–1.2 μ in diameter) mycelium. The mycelium is nonseptate, of a strong yellow color, and spore formation in or on the host is not seen. The organism undoubtedly belongs to the chromogenic actinomycetes, of the genus *Streptomyces.*

Mycorrhiza Formations

The associations of certain actinomycetes with the root systems of certain plants are of particular interest. These associations are believed to be comparable to mycorrhiza formations by true fungi. Peklo (1910) made a detailed study of the endophytes of the alder bush, *Alnus glutinosa,* and of sweet gale, *Myrica gale.* Two species of actinomycetes, *A. alni* and *A. myricae,* were isolated. These organisms produced, in culture, swellings comparable to those formed by animal pathogens. The significance of these associations for plant growth has not been fully elucidated.

Formation of small tubers on the roots of the oleander has been demonstrated by Roberg (1934). The organism is similar to *A. alni* and was described as *A. elaeagni.*

All three of these actinomycetes definitely belong to the genus *Nocardia.* Only one of them is included in the classification, namely, *N. alni.*

A further study of these associations has been made by von Plotho (1941). It was suggested (Lieske) that the role of actinomycetes consists in enabling the plant to fix atmospheric nitrogen.

Actinomycetes and Plant Development

Lutman suggested that the occurrence of actinomycetes in the outer layers of roots and tubers of the potato plant corroborates his theory that these organisms play an important role in plant growth. The stems above ground are also infected; but the tips of young roots and stems contain only a few strands between the cells. These facts suggested that the infection is systemic and hereditary. Young potato plants grown from disinfected seed and in disinfected soil are found to contain numerous actinomycete filaments.

According to Lutman, potato scab lesions are associated with strands of actinomycetes extending from the abnormal cells of the cork cambium to the interior of the tuber. Similar strands have been found in clean tubers grown on land never known to produce scabby tubers. The strands found under the scabs seem to be unusually large and numerous, especially those about five to ten cells below the pathological tissue.

The cell walls of Jerusalem artichoke tubers and the enlarged roots of beets, carrots, parsnips, and turnips contain gram-positive filaments which seem to be of the same sort as those occurring in the potato plant. The suggestion was made that since actinomycetes are abundant in the roots of plants, they may take part in the synthesis of alkaloids and proteins. Since large numbers of soil actinomycetes are pectin-dissolving, the different varieties found in the various host plants may be only modifications of one large species. The walls of higher plants were believed to be living, through the presence and action of strands of actinomycetes. The effects of actinomycete filaments surrounding every cell suggested the theory that the materials they withdraw from the cells and the products which they excrete and which must be absorbed by the cells change the characteristics of the cells.

Richards outlined in detail the method of staining the potato scab organism. The organism can be selectively impregnated with carbol-auramin and when exposed to ultraviolet radiation, it fluoresces bright yellow. The hyphae are stained bright yellow. This permits ready localization and study of the micropathology of the tissue with a simple fluorescence microscope. The staining technic is done at room temperature. No counterstain is used. The results obtained tended to confirm Lutman's conclusion that the filaments are intercellular and grow within the middle lamellae. After complete removal of the paraffin, the sections are strained 4 minutes in carbon-auramin (distilled water 97 ml, liquefied phenol 3 ml, certified auramin 0.1 gm), washed, destained in a 0.5 per cent solution of NaCl in 70 per cent alcohol with 0.5 ml of HCl (conc.) per 100 ml, washed, and mounted in glycerol.

Vörös *et al.* reported that streptomycin exerts a protective effect upon the potato plant, rendering it resistant to *Phythophtora,* via the polyphenol-polyphenolase system of the host plant. The fact that polyphenolases are copper enzymes, their activity depending upon the copper supply of the plant, and the fact that copper and streptomycin were found to exert a synergistic effect may help to explain the above effect of streptomycin

EPILOGUE TO VOL. I

These then are the actinomycetes, dismissed only about two decades ago as a "little-known group of microorganisms," and considered by some as fungi and by others as bacteria. I have presented in these pages my personal experiences with the actinomycetes, especially their occurrence in nature, their structure and functions, and their role in natural processes. I have attempted to summarize some of the reactions whereby they carry out their characteristic biochemical activities, their varied biochemical potentialities, which are at present being taken advantage of for the benefit of the human race. The subsequent volumes will deal with the problems of how to recognize them and how to utilize them for the production of valuable drugs that are saving millions of human lives.

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INDEX TO SPECIES OF ACTINOMYCETES

Actinobacillus (*Bacillus*) *oligocarbophilus*, 130
Actinomyces
  albido-flavus, 5
  albus, 11, 42, 43, 49, 50, 62, 70, 156
  alni, 275, 276
  arborescens, 5
  asteroides, 5, 52, 70
  bovis, 5, 7, 8, 13, 29, 44, 47, 51, 52, 57, 61, 62, 70, 80, 87, 88, 102, 132, 166, 252, 254, 255, 257–261
  bovis albus, 5
  bovis farcinicus, 5
  bovis luteo-roseus, 5
  bovis sulphureus, 5
  brasiliensis, 250
  canis, 5
  caprae, 52
  carneus, 5
  cati, 5
  chromogenes, 42, 70
  chromogenus, 5, 49, 210, 265
  citreus, 5
  cloacae, 41
  cuniculi, 5
  cyaneus, 199
  discofoliatus, 263
  elaeagni, 276
  elastica, 155
  ferrugineus, 5
  flavus, 50
  Foersteri, 5
  fuscus, 155
  graminis, 4
  hanseni, 40
  Hoffmanni, 5
  invulnerabilis, 136
  israeli, 7, 44, 52, 70, 87, 129, 166, 264
  keratolytica, 256
  madurae, 52
  monosporus, 135
  mutabilis, 104
  myricae, 275
  naeslundii, 129
  nocardi, 52
  odorifer, 42, 49
  oligocarbophilus, 42, 130, 132
  pelogenes, 38
  pleuriticus canis familiaris, 5
  pluricolor, 5
  poolensis, 275
  pox, 275
  roseolus, 156
  scabies, 212, 265
  spinae, 157
  sulphureus, 4
  thermophilus, 135
  tumuli, 274
  verrucosus, 156
  vesicae, 52
  violaceus, 5, 50
Bacillus
  farcinicus, 5
  oligocarbophilus, 114, 130
Chainia antibiotica, 66
Cladothrix asteroides, 5, 49, 261
Leptothrix buccalis, 3
Microbispora rosea, 64
Micrococcus cytophagus, 188
Micromonospora
  caballi, 256
  chalcea, 93, 188
  fusca, 93
  propionici, 155
  vulgaris, 93, 135
Micromyces Hoffmanni, 5
Mycobacterium
  butyricum, 68
  chalcea, 188
  eos, 217
  fortuitum, 191
  leprae, 68
  phlei, 68, 191, 229
  rhodochrous, 191, 192
  smegmatis, 68, 191, 229
  stercoris, 68
  tuberculosis, 209, 214, 215, 219, 229, 230, 234, 235
Mycococcus cytophagus, 188

Nocardia
- *actinomyces*, 62
- *alba*, 184
- *alni*, 276
- *aquosa*, 152
- *asteroides*, 67–69, 81, 84, 90, 120, 124, 127, 159–162, 170, 178, 181, 184, 191, 192, 254, 255, 258, 261, 262, 264
- *atlantica*, 125
- *blackwellii*, 67–69
- *brasiliensis*, 67, 68, 124, 127, 181, 191, 262, 264
- *butyricum*, 67
- *calcarea*, 157
- *caprae*, 68, 69, 262
- *caviae*, 68
- *cellulans*, 33, 157
- *citrea*, 169, 188
- *coeliaca*, 152
- *convoluta*, 68, 69
- *convolutus*, 160
- *corallina*, 67–69, 91, 102, 120, 156, 158, 160, 189, 192, 200
- *cuniculi*, 38, 67–69
- *eppingeri*, 263
- *erythropolis*, 67–69, 152
- *farcinica*, 55, 61, 62, 160, 175, 182, 261, 262
- *flava*, 125, 184
- *foersteri*, 62
- *gardneri*, 68
- *globerula*, 67–69, 152
- *intracellularis*, 67, 68
- *leishmanii*, 67, 68
- *leprae*, 67
- *madurae*, 67, 68, 124, 127, 181, 191, 261
- *minima*, 67–69
- *opaca*, 67–69, 152, 153
- *paraffinae*, 67–69, 160
- *paraguayensis*, 180, 181
- *pelletieri*, 67, 68, 124, 127, 192
- *petroleophila*, 130
- *polychromogenes*, 67–69, 90, 101, 154, 160–162, 170, 172, 199
- *pretoriana*, 258
- *rangoonensis*, 67, 68
- *restricta*, 152
- *rhodnii*, 45, 195
- *ruber*, 184
- *rubra*, 68, 69, 160–162, 170, 172
- *rugosa*, 192, 194, 195
- *sebivorans*, 135
- *somaliensis*, 124, 127
- *sylvodorifera*, 68, 69
- *viridis*, 184

Oospora
- *cretacea*, 274
- *Guignardi*, 5
- *intermedia*, 274
- *Metschnikowi*, 5
- *nigrificans*, 274
- *rosella*, 274
- *scabies*, 7, 8, 48, 265
- *tenax*, 274
- *violacea*, 274

Proactinomyces cytophagus, 189

Streptomyces
- *albido-flava*, 42
- *albidoflavus*, 146
- *alboflavus*, 155, 160
- *albogriseolus*, 236
- *albus*, 38, 42, 44, 112, 121, 125, 135, 152, 154, 155, 160, 167, 173, 174, 188, 193, 195, 222
- *albus* var. *ochraceus*, 209
- *antibioticus*, 20, 125, 160, 189, 222, 226, 228, 231
- *aurantiaca*, 42
- *aurantiacus*, 156
- *aureofaciens*, 105, 115, 116, 123–125, 145, 152, 160, 164, 177, 189, 193, 194, 223, 224, 237
- *aureus*, 68, 154, 160, 185, 186
- *autotrophicus*, 130
- *bikiniensis*, 141, 178
- *bobiliae*, 38, 121, 160–162, 170, 172, 185
- *cacaoi*, 43, 179
- *californicus*, 120, 122, 160, 236
- *candidus*, 179
- *carnea*, 42
- *cellulosae*, 185
- *chromogena*, 42
- *chrysomallus*, 116, 160, 231
- *citreus*, 154
- *clavifer*, 266
- *coelicolor*, 51, 100, 101, 104, 105, 110–112, 115, 121, 122, 131, 145, 148, 156, 158, 160, 170, 188–190, 197, 199, 200, 220, 222, 256
- *craterifer*, 160
- *cyaneus*, 181
- *diastaticus*, 125, 179, 187
- *diastatochromogenus*, 185
- *erythreus*, 127, 160, 165, 190, 238
- *erythrochromogenus*, 121, 185
- *exfoliatus*, 186
- *flaveolus*, 105, 108, 121
- *flavovirens*, 20
- *flavus*, 160, 220, 266
- *fragilis*, 123
- *floridae*, 120, 122, 236
- *fradiae*, 54, 110–112, 116, 126–129, 141–145, 150, 152, 154, 158–162, 170, 171, 185, 186, 190, 193, 194, 223, 224, 226, 230, 236, 237
- *fulvissimus*, 160, 185
- *gardneri*, 20

gelaticus, 121, 152
globisporus, 108, 157, 192, 197, 222
globisporus vulgaris, 157
globosus, 108, 157, 160
griseoflavus, 185
griseolus, 121, 186
griseoviridis, 123
griseus, 20, 66, 68, 74, 77, 78, 87, 96, 98, 104–107, 110–112, 115–117, 119–123, 125, 127, 129, 131, 138–143, 145, 147–155, 157–162, 164, 167, 170–172, 175, 176–179, 181, 185–187, 189, 190, 193, 194, 196, 197, 202, 203, 222–226, 230, 233, 235, 240–242
griseus var. *purpureus*, 236
halstedii, 185
humidus, 235
intermedius, 273
ipomoea, 160, 275
kanamyceticus, 236
lavendulae, 20, 50, 105, 106, 123, 125, 128, 129, 145, 150, 152, 153, 157, 158, 160–162, 167, 178, 185, 222, 225, 232
lipmanii, 160
longisporus, 222
longisporus ruber, 156
nigrificans, 209
madurae, 261, 262, 264
marinus, 125
nigra, 42
niveus, 238
oligocarbophilus, 146
olivaceus, 121, 143, 160, 179, 181, 185, 193, 194, 230
olivochromogenus, 121
omiyaensis, 236
parvullus, 231
parvus, 160
pelletieri, 262, 264
phaeochromogenes, 127
pheochromogenus, 121, 185
poolensis, 121, 230
praecox, 160, 210, 211, 273
puniceus, 122, 236
purpurescens, 160
reticuli, 83, 99, 160
reticuloruber, 83
rimosus, 59, 111, 145, 154, 160, 165, 206, 223, 226, 237
roseochromogenes, 127, 154, 160–162
roseochromogenus, 121
roseodiastaticus, 202
roseus, 222
ruber, 68, 121, 202, 222
rubescens, 38, 152
rubrireticuli, 58, 155
scabies, 104, 106, 144, 149, 155, 160, 175, 179, 190, 191, 197, 210, 211, 218, 265, 267, 268, 270–273, 275
somaliensis, 262, 264
spectabilis, 238
spheroides, 238
sterilis, 105
tricolor, 199, 209
venezuelae, 68, 112, 116, 130, 131, 145, 152, 160, 181, 236
verne, 200
verticillatus, 21
vinaceus, 122, 226
violacea, 42
violaceoruber, 199, 200
violaceus, 105, 136, 155, 160, 173, 185, 200, 222
violaceus niger, 201
violaceus-ruber, 185
viridis, 200, 273
viridochromogenes, 160, 189, 192, 193, 200
Streptothrix
  actinomyces, 7, 51
  alba, 30, 42, 62, 70
  albido-flava, 5
  Albus, 5
  aurantiaca, 51
  carnea, 5
  chromogena, 9
  chromogenus, 5
  cuniculi, 5
  eppingeri, 51
  Eppingerii, 5
  erysipeloides, 51
  erythrea, 41
  flava, 70
  Foersteri, 2, 5, 47, 62
  israeli, 7, 254
  leucea, 41
  leucea saprophytica, 41
  madurae, 51
  mihi, 51
  niger, 5
  odorifera, 118
  rubra, 51
  violacea, 5, 70
Thermoactinomyces
  monosporus, 63
  thalpophilus, 63
  vulgaris, 40, 63
Thermopolyspora bispora, 64
Waksmania rosea, 64

GENERAL INDEX

- Acetate, utilization by cells of *Streptomyces griseus*, 150
- Acid
  - production, 157, 158
  - reaction of *Streptomyces griseus*, as affected by metals, 141
  - utilization, 158
- *Actinomyces*, historical significance of name, 52
- *Actinomycetales*, classification and description, 56, 65
- Actinomycetin, action on bacteria, 173, 174
- Actinomycins, production and properties, 203, 231
- Actinomycosis, 1, 7, 47, 251, 257
- Actinophage
  - effect on
    - *Antinomycetales*, 181
    - plaque formation, 176
    - streptomycin production, 176, 177
  - isolation from soils, 179, 180
  - mechanisms, 175–181
  - production, 175
- Actinorhodin, 205
- Actinorubin, 142
- Adaptation to antimicrobial agents, 109
- Aerobic actinomycetes, metabolism, 129, 130
- Aldolase, 189
- Alginase, 188
- Allergies caused by acinomycetes, 263
- Amino acid
  - content of cell walls, 162, 163
  - synthesis, 163
- Amylase
  - hydrolysis of mannosidostreptomycin, 187
  - production, 187
- Anaerobic actinomycetes, metabolism, 131, 149
- Antagonisms in mixed populations
  - forced, 209
  - types, 207
- Antagonistic organisms
  - isolation from natural substrates, 211, 213, 215
  - soil as source, 209, 211–215
- Antagonistic properties
  - antiphage, 219
  - bactericidal, 208, 218
  - distribution among actinomycetes, 210, 213
  - effects on pathogenic organisms, 209, 211, 216–218, 220
  - of fungi and bacteria, effects on actinomycetes 221
  - fungicidal, 208, 209, 215–218, 220
  - relation to pigmentation, 217
  - screening methods to determine, 212
- Anthocyanins, 200, 201
- Antibacterial substances
  - as chemotherapeutic agents, 208
  - production by organisms, 207, 208
- Antibiotic-producing organisms
  - activity of soil actinomycetes, 211–215
  - isolation and testing 226, 227
  - mutations caused by irradiation, 107
  - variants, 105
- Antibiotics
  - activity against pathogens, 241
  - antiphage activity, 24, 219
  - antiprotozoan, 240
  - antitumor, 240
  - antiviral, 219, 240
  - autoantagonisms, 222, 223
  - bactericidal, 216
  - biosynthesis, 163, 241, 242
  - chemical structure, relation to activity, 242
  - classification, 204, 229
  - concentration, effect on resistance, 238, 239
  - effect
    - of metals on production, 142, 145
    - of phages on production, 177, 178, 181
  - historical studies on production, 14
  - identification by bacterial stains, 240
  - isolation and testing methods, 211
  - media for production, 229
  - principles of production, 225
  - production in natural substrates, 45, 221
  - properties, 207
  - screening programs, 211, 217, 241
  - spectra, 228–230, 241
  - test organisms, 218, 219
- Antifungal action, 197, 232
- Antigenic properties, 166–167
- Antimicrobial activity of streptomycetes, as affected by metals, 142
- Antimicrobial spectra of *Streptomyces fradiae* strains, 237
- Antiphage factors, 24, 219

Antitumor activity of actinomycetes, 219, 240
Aryl sulfatase production, 191, 192
Aureothricin, 205
Autoinhibition, 222, 224, 227
Autolysis
  mechanisms, 168
  relation to antibiotic production, 171
Autotrophy, 130

B_{12} production, *see* Vitamin B_{12} production
Bacteria, relation to actinomycetes, 1, 13, 53, 71
Bacteriolysis, mechanisms, 171
Biochemistry, historical studies, 14
Biology, historical studies, 11
Biotin production by *Streptomyces olivaceus*, 194
Bromtetracycline, 145

Cancer, actinomycins in treatment, 203
Candicin, production as affected by metals, 142
Carbohydrate composition of cell walls for taxonomic use, 160
Carbomycin, 238
Carbon-dioxide fixation by anaerobic actinomycetes, 149
Carbon sources, utilization
  agar, 125
  by antibiotic producers, 122, 123
  as basis of classification for antibiotic-producing streptomyces, 121
  cellulose, 124
  chitin, 125
  efficiency and metabolic changes, 125
  glucose, 132, 133
  of proteins, 124
  relation
    to nitrogen utilization, 128
    to streptomycin production, 119, 133
  in species characterization, 119, 120
  unusual compounds, 125
  by various actinomycetes, 118, 120, 122, 123
Carotinoids, 193, 202
Catalase activity, relation to streptomycin production, 190
Cell walls, chemical composition, 158–163
Cellulase production, 188
Cellulose decomposition, 155, 244
Chitin decomposition, 155
Chitinase production, 186
Chloramphenicol, production and properties, 145, 236
Chlortetracycline, production and properties, 145, 164, 237
Cholic acid utilization, 152
Chromopars, 199
Chromophores, 199
Classification systems
  carbon utilization by streptomyces, 20, 201
  cell wall composition, 69
  Buchanan's, 56
  generic, 47
  group characteristics, 67–70
  group identification by sporulation, 67
  historical background, 55
  pigmented antibiotics, 204
  specific, 49
Club formation, 7, 81
Coelicolorin, 205
Coenzyme A production, 192, 195
Colony
  formation, 8, 76
  variants of *Streptomyces griseus*, 96
Constancy of characters, 95
Coremia formation, 80, 84
Cortexone conversion, 152
Crop yields in relation to actinomycete population of soil, 33
Cross-resistance, 222, 223
Cultivation of actinomycetes, techniques, 17
Cultural variations, 103

Decomposition of plant materials
  cellulose, 155, 244
  complex, 247
  hemicelluloses, 155, 244, 246
  humus, 247
  lignin, 244, 247
  polysaccharides, 244–246
  proteins, 245
  thermophilic composts, 248
Deguanidases, 186
Diastase production, 185
Diketopiperazine, effect on rice germination, 196
Diseases
  of animals
    actinomycetes isolated from, 252–257, 262
    actinomycosis, 7, 251–253, 257
    allergic reactions, 263
    mycetoma, 261, 262
    nocardiosis, 261, 263, 264
    therapy, 263
  causation, 6, 13
  of plants
    caused by chromogenic organisms, 275
    citrus gummosis, 275
    mangel scab, 274
    necrosis of seedlings, 275
    potato scab, 7, 265–274
    strawberries, 275
    sugar beet scab, 274
    sweet potato pox, 275

Distribution of actinomycetes in
  air, 41
  animal tissues, 6, 44
  foodstuffs, 42
  fresh waters, 38, 40
  geologic formations, 45
  manures and composts, 10, 40
  plants, 43
  sea water and marine sediments, 37
  soil, 9, 12, 30–32, 34–36
Drying, influence on actinomycetes, 135

Enolase production by *Streptomyces coelicolor*, 189
Enzyme systems of *Nocardia corallina*, 189, 192
Enzymes, extracellular and endocellular, 183
Erythromycin, 238
Esterase, 189
Ethanol dehydrogenase production by *Streptomyces coelicolor*, 189

Fermentation
  of glucose, 149, 150, 157
  by *Streptomyces griseus*
    carbon balance, 151
    nitrogen balance, 154
F-forms, 101
Flavoprotein production, 193
Foodstuffs, actinomycetes in, 42
Fungi, relation to actinomycetes, 1, 13, 53, 71

Generic interaction in *Streptomyces*, 110
Grisein production, 142, 145, 195
Growth
  as affected by salt concentrations, 146, 147
  -promoting effects on
    animals, 195, 196
    bacteria, 196
  in soil, as affected by rye straw and dried blood, 246, 247
  of various genera
    Actinoplanes, 62
    Micromonospora, 61
    Nocardia, 48
    Streptomyces, 50, 51, 54, 55, 58, 59, 138–145
    Streptosporangium, 63
Glucose utilization, effect on
  acidity, 132
  streptomycin production, 133

Hemicellulose decomposition, 155, 244, 246
Hemolysin production, 181
Heterokaryosis in *Streptomyces*, 110–112
Humus, formation and decomposition, 247, 248
Hydroactinochromes, 201

Identification of species
  morphological properties in, 18
  physiological and cultural properties in, 19
  techniques, 17, 22
Invertase production, 185, 188
Irradiation, effect on
  mutations, 107
  vitamin B_{12} production, 194
Isolation techniques, 17

Keratin decomposition, 153
Keratinase production, 185

Lab production, 185
Laccase, 189
Laminarinase, 188
Leucocyte-stimulating factor produced by *Streptomyces griseus*, 193
Life cycle
  Actinomyces bovis, 87
  Micromonospora, 94
  Nocardia, 89, 90, 91
  Streptomyces, 75
Light, influence, 136
Limestone precipitation, 156
Lipase, 189
Lipoactinochromes, 202
Luminescence, relation to antibiotic activity, 20
Lysis of *Streptomyces fradiae*, as affected by calcium, 145
Lysozyme
  action on cell walls, 159, 160, 170, 172, 173
  enzymatic properties, 183

Macrolides, 238
Magnamycin, 238
Mangel scab, 275
Mannosidostreptomycin, hydrolysis by amylase 187
Mannosidostreptomycinase, 187
Media for growth of actinomycetes, 18
Melanin, 203
Mesophilic organisms, 134
Metabolic processes of *Streptomyces*, phases, 116
Metabolic reactions, role of metallic elements 146, 147
Metabolism
  of aerobic actinomycetes, 129, 130
  of anaerobic actinomycetes, 131, 149
  carbohydrate, by *Streptomyces*, 148, 149
  fatty acids, by *Nocardia opaca*, 152, 153
  historical studies, 114
  role of antibiotic biosynthesis in, 241, 242
Methymycin, 238
Mineral requirements of *Streptomyces griseus*, 13

- Morphological characteristics
  - *Actinomyces*, 86
  - constancy in species and genera, 86
  - in identification, 18
  - *Micromonospora*, 79, 93
  - *Nocardia*, 88
  - relation to those of bacteria and fungi, 13
  - *Streptomyces*, 92
  - variations in, 101
- Motility, 81
- Mutations
  - as affected by irradiation, 107
  - as affected by physical and chemical agents, 109
  - development of phage-resistance, 110
  - genetic studies on *Streptomyces*, 110
  - saltations, 106
- Mycelium,
  - aerial
    - coremia, 84
    - formation, as affected by medium, 67
    - pigmentation, 83
    - secondary, 84
    - structure and growth, 75, 82
    - verticil formation, 21, 83
    - zonation, in *Streptomyces* colony, 25
  - substrate
    - structure and growth, 73, 74, 75, 80
    - suspensions, as affected by shaking, 25
- Mycetoma, 261, 262, 264
- Mycolysate, clinical use, 209
- Mycorrhizal associations, 275

- Neomycin production
  - and properties, 230, 236, 237
  - relation to autolysis, 171
  - by *Streptomyces fradiae*, as affected by metals, 142
- Neomycin B, effect on growth of broad beans, 196
- Niacin production by *Streptomyces olivaceus*, 194
- Nitrate reduction, 156, 157
- Nitrification, 156
- Nitrogen
  - fixation, 157
  - sources, utilization
    - amino acids, 126
    - plant proteins, 127
    - relation to antibiotic production, 124, 127
    - relation to carbon utilization, 128
    - by various actinomycetes, 122, 126
- Nocardiosis, 261, 263, 264
- Nomenclature
  - *Actinomyces*, historical significance, 52
  - generic, systems, 47
  - specific, 49
  - *Streptothrix*, historical significance, 51

- Odor production, 43, 156, 250
- O-forms, 96
- Oleandomycin, 238
- Oxidation of steroids and lipids, 152
- Oxygen consumption, 129
- Oxytetracycline, production and properties, 165, 237

- Pantothenic acid, production by *Streptomyces olivaceus*, 194
- Paraffin decomposition, 156, 247
- Parasitic actinomycetes, 251
- Pathogenicity, variability of *Streptomyces scabies*, 104; *see also* Diseases of animals, Diseases of plants
- Penicillinase production, 191
- Phage, effect on lytic properties, 110
- Phenol oxidases, 189
- Phosphatase production by anaerobic actinomycetes, 192
- Phosphofructokinase production by *Streptomyces coelicolor*, 189
- Phosphoglyceryl kinase production by *Streptomyces coelicolor*, 189
- Physiological variations, 103
- Picromycin, 238
- Pigment production
  - as affected by environment, 203
  - relation to antibiotic production, 198, 202–204
  - relation to antimicrobial activity, 217
  - by *Streptomyces*, as affected by metals, 139, 144
  - by tetracyclines, 206
  - use in classifying actinomycetes, 198
- Pigments
  - anthocyanins, 200
  - brown-black, 202
  - carotinoids, 202
  - chemical and physical properties, 198, 200
  - chromopars, 199
  - chromophores, 199
  - green, nature of, 200
  - hydroactinochromes, 201
  - lipoactinochromes, 202
  - prodigiosin, 202
  - rhodomycin, 202
  - solubility, 199
  - use in classifying antibiotics, 204
  - variations, 103, 105
- Plant
  - development, as affected by actinomycetes, 276
  - residues, decomposition, 243
- Poisons, antimicrobial effects, 137
- Polyene antibiotics, 238–240
- Polysaccharidase production, 188

Population studies, methods
  microscopic, 20
  plate, 24
  selective culture, 25
Porphyrin biosynthesis, 194, 195
Potato scab
  biological control, 272
  causative organisms, 265
  control by *Streptomyces praecox*, 210, 211
  environmental effect, 267–270, 272
  relation of host to parasite, 270
  treatment, effect of, 271
Preservation of cultures, methods, 26, 128
Prodigiosin pigments, 202
Progesterone metabolism, 152
Protease production, 140, 183, 185
Proteolytic activities, 153
Psychrophilic organisms, 134
Pyocyanase, 207
Pyridoxine, production by *Streptomyces olivaceus*, 194

Relation of actinomycetes to bacteria and fungi, 1, 13, 53, 71
Rennet production, 185
Reproduction, methods, 75, 78, 84, 86
Resistance to metabolic products and antimicrobial substances, 109
Respiration of *Nocardias*, carbohydrates in, 151
R-forms, 96
Rhodomycin, 202
Riboflavin production, 193, 194
Rubber decomposition, 155, 247

Saprophytic actinomycetes, 251, 255
Sarkomycin, effect on growth of broad beans, 196
Scab, *see* Potato scab, Sugar beet scab, Mangel scab
Sclerotia formation in a streptomyces, 81
Screening methods
  antifungal surveys, 215
  relation between antimicrobial activity and pigmentation, 217
  for specific antibiotic producers, 217
  in study of antagonistic properties, 211
  test organisms, 216, 219
Sectoring, 98, 107
Serological relations among actinomycetes, 166, 167
Sexuality, 74
S-forms, 95
Soil
  actinomycetes
    antagonistic, 209, 211–215, 222
    factors controlling, 37
    seasonal variation, 30, 34
    variation with depth, 31
  enrichment with pathogens, 222, 223
  processes, role of actinomycetes in, 9
Spiromycin, 238
Spoilage, agents of, 250
Spores
  chains, 85
  distribution of types among groups of *Strept*
  myces, 86
  formation of different types, 71, 77, 79, 82, 84
  germination, 19, 73
  role in characterization of species, 85
Sporophores of *Streptomyces*, 54, 55, 58–60, 82, 83
Sporulation, as affected by cobalt, 143
Staining, 77
Starch decomposition, 155
Steroid oxidation, 191
Stimulants, microbial, 137
Streptomycin
  antibacterial activity, 230
  biosynthesis, 163–166
  destruction by deguanidases, 186
  effect on *Mycobacterium tuberculosis*, 234
  production
    as affected by actinophage, 176, 177
    as affected by catalase activity, 190
    by *Streptomyces bikiniensis*, 141
    by *Streptomyces griseus*, 115, 119, 133, 138–14
    145, 151
    relation to autolysis, 171
  properties, 230, 233
  protective effect on potato plant, 276
Streptothricin, production and properties, 222
Streptothrix, historical significance of name, 51
Streptovaricin, production and properties, 238
Sugar beet scab, 274
Sweet potato pox, 275
Symbiosis with insects, 195

Temperature, influence, 133
Tetracyclines
  pigment production in presence of metals, 206
  production and properties, 237
Thermophilic organisms, 134
Thiamin production, 193, 194
Thioaurin, 205
Thiolutin, 205
Triose phosphate dehydrogenase, production b
  Streptomyces coelicolor, 189
Triose phosphate isomerase, production by *Stre*
  tomyces coelicolor, 189
Tyrosinase, 191

traviolet radiations
  antibacterial action, 136
  mutagenic and lethal effects, 102, 103, 105, 107
rea production from guanidine, 186
rease production, 186

ariations, 97–99, 101, 103, 110
iomycin, production and properties, 122, 236
itamin B_{12} production
  factors affecting, 142, 143, 145, 193, 194
  relation to antibiotic production, 193
Vitamin synthesis, relation to growth-stimulating effect, 193, 195

Wool digestion, as affected by magnesium, 186

Xanthomycins A and B, 205
Xylanase, 188

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